



## Review

# Interaction of antimicrobial peptides with biological and model membranes: structural and charge requirements for activity

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## Abstract

Species right across the evolutionary scale from insects to mammals use peptides as part of their host-defense system to counter microbial infection. The primary structures of a large number of these host-defense peptides have been determined. While there is no primary structure homology, the peptides are characterized by a preponderance of cationic and hydrophobic amino acids. The secondary structures of many of the host-defense peptides have been determined by a variety of techniques. The acyclic peptides tend to adopt helical conformation, especially in media of low dielectric constant, whereas peptides with more than one disulfide bridge adopt  $\beta$ -structures. Detailed investigations have indicated that a majority of these host-defense peptides exert their action by permeabilizing microbial membranes. In this review, we discuss structural and charge requirements for the interaction of endogenous antimicrobial peptides and short peptides that have been derived from them, with membranes. © 1999 Elsevier Science B.V. All rights reserved.

**Keywords:** Antimicrobial peptide; Amphiphilicity; Alpha helix; Beta structure; Cationic charge; Membrane permeabilization

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## 1. Introduction

A large number of proteins that play a crucial role in cellular physiology are in intimate association with lipid components of cellular membranes through interactions facilitated by peptide segments composed predominantly of apolar amino acids [1,2]. There have been extensive efforts to determine the features in peptide segments that would favor membrane association and also the orientation of peptide segments in the bilayer as well as effects of membrane-association on bilayer structure [3–8]. Peptide antibiotics which are hydrophobic in nature such as gramicidin A [9,10] and alamethicin [11,12] have been extensively used to delineate peptide–membrane interactions. Their small size and easy availability have made it possible to study lipid–peptide interactions in depth by a variety of biophysical techniques. Peptides like gramicidin A and alamethicin were found to have the ability to modulate ion flux across membranes and hence became popular models for understanding how ion channel proteins function [9–13]. In the extensive studies that have been reported, the emphasis was on lipid–peptide interactions rather than their biological activities. Since the early 1980s it has become evident that peptides are an important constituent of the host-defense mechanism against microbes in species right across the evolutionary scale from insects to mammals [14–22]. The amino acid composition of these peptides, along with the presence of cationic amino acids, suggested that they would have the ability to associate with membrane lipids. Subsequent studies indicated that these peptides, in spite of considerable variation in primary

structure and length, exerted their biological activities by permeabilizing bacterial membranes, unlike therapeutically used antibiotics [23–28]. To date, the primary structures of a large number of endogenous host-defense peptides have been determined. The genes encoding these peptides have been characterized and so also their expression profiles [15–22]. Structure–function studies on these peptides have provided considerable insights into how this class of peptides, which are not structurally constrained like gramicidin A and alamethicin, modulate membrane structure and also show specificity with respect to target cells [15–27]. In this review, we discuss the structural and charge requirements for the interaction of antimicrobial peptides with biological and model membranes in relation to the biological activities that have emerged from extensive investigations in this area. It has also been possible to generate variants of endogenous host-defense peptides much shorter in length than the parent peptides and designer peptides engineered to have comparable or even improved activities and specificity as compared to endogenous peptides, on the basis of biophysical principles. These short peptides appear to effectively interact with membranes and exert their activities in a manner similar to the longer peptides. We focus our attention here on short peptides that have been derived from host-defense peptides and engineered in order to obtain an insight into physico-chemical properties required for activity. We also examine some of the recent work on magainins, cecropins and defensins, based on which detailed models for their interaction with membranes have been proposed, with a view to explore whether a unified mod-

el for the mechanism of peptide antibiotics can be proposed.

## 2. Primary and secondary structures of endogenous antibacterial peptides

The primary structures of host-defense antimicrobial peptides have been extensively documented in several reviews [14–24], and are also available on web sites. Hence, we have not provided an extensive documentation in this review. However, in Table 1,

sequences of a few antibacterial peptides from different species are presented in order to highlight the extensive differences in primary sequences, positioning of charged residues, number of disulfide bridges and number of residues. It is apparent that all these peptides would be cationic at neutral pH, existing either as the free acid or as the amide derivatives. The defensins and other cyclic peptides have R as the predominant cationic residue unlike cecropins and magainins. The number of S–S bridges also varies between one to four. Pardaxin and melittin, though originally identified as hemolytic peptides

Table 1  
Primary structures and activities of endogenous peptides possessing antibacterial and hemolytic activities<sup>a</sup>

Peptide	Sequence	Remarks
Cecropin A (Insect)	KW <b>KLF</b> <u><b>K</b></u> <b>K</b> <u><b>E</b></u> <b>KV</b> <u><b>G</b></u> <b>Q</b> <u><b>N</b></u> <b>I</b> <u><b>R</b></u> <u><b>D</b></u> <b>G</b> <b>I</b> <b>K</b> <b>A</b> <b>P</b> <b>A</b> <b>V</b> <b>V</b> <b>G</b> <b>Q</b> <b>A</b> <b>T</b> <b>Q</b> <b>I</b> <b>A</b> <b>K</b> -Am	Net positive charge vary between 2-7 In cecropins. Distribution of charges are similar. Activity <i>E. coli</i> > <i>P. aeruginosa</i> >> <i>S. aureus</i>
Magainin 2 (amphibian)	G <b>I</b> <b>G</b> <b>K</b> <b>F</b> <b>L</b> <b>H</b> <b>S</b> <b>A</b> <b>K</b> <b>K</b> <b>F</b> <b>G</b> <b>K</b> <b>A</b> <b>F</b> <b>V</b> <b>G</b> <u><b>E</b></u> <b>I</b> <b>M</b> <b>N</b> <b>S</b>	Active against <i>E. coli</i> and not <i>P. aeruginosa</i> or <i>S. aureus</i>
CPF 1 (amphibian)	G <b>F</b> <b>A</b> <b>S</b> <b>F</b> <b>L</b> <b>G</b> <b>K</b> <b>A</b> <b>L</b> <b>K</b> <b>I</b> <b>G</b> <b>A</b> <b>N</b> <b>A</b> <b>L</b> <b>G</b> <b>G</b> <b>A</b> <b>P</b> <b>Q</b> <b>Q</b>	Activity: <i>S. aureus</i> ~ <i>P. aeruginosa</i> > <i>E. coli</i>
PGLa(amphibian)	G <b>M</b> <b>A</b> <b>S</b> <b>K</b> <b>A</b> <b>G</b> <b>I</b> <b>A</b> <b>G</b> <b>K</b> <b>I</b> <b>A</b> <b>K</b> <b>V</b> <b>A</b> <b>L</b> <b>K</b> <b>A</b> <b>L</b> -Am	Activity: <i>E. coli</i> > <i>P. aeruginosa</i> > <i>S. aureus</i>
Bombinin BLP-1 (amphibian)	G <b>I</b> <b>G</b> <b>A</b> <b>S</b> <b>I</b> <b>L</b> <b>S</b> <b>A</b> <b>G</b> <b>S</b> <b>A</b> <b>L</b> <b>K</b> <b>L</b> <b>A</b> <b>G</b> <b>L</b> <u><b>A</b></u> <b>E</b> <b>H</b> <b>F</b> <b>A</b> <b>N</b> - Am	Activity : <i>C. albicans</i> > <i>E. coli</i> > <i>S. aureus</i> > <i>P. aeruginosa</i>
Pardaxin(fish)	G <b>F</b> <b>F</b> <b>A</b> <b>L</b> <b>I</b> <b>P</b> <b>K</b> <b>I</b> <b>S</b> <b>S</b> <b>P</b> <b>L</b> <b>F</b> <b>K</b> <b>T</b> <b>L</b> <b>S</b> <b>A</b> <b>V</b> <b>G</b> <b>S</b> <b>A</b> <b>L</b> <b>S</b> <b>S</b> <b>G</b> <u><b>E</b></u> <u><b>Q</b></u> <u><b>E</b></u>	Activity: broad spectrum, G+ and G-
Melittin (bee venom)	G <b>I</b> <b>G</b> <b>A</b> <b>V</b> <b>L</b> <b>K</b> <b>V</b> <b>L</b> <b>T</b> <b>T</b> <b>G</b> <b>L</b> <b>P</b> <b>A</b> <b>L</b> <b>I</b> <b>S</b> <b>W</b> <b>I</b> <b>K</b> <b>R</b> <b>K</b> <b>R</b> <b>Q</b> <b>Q</b> -Am	Activity: <i>E. coli</i> > <i>S. aureus</i> > <i>P. aeruginosa</i>
Seminalplasmin (bovine)	S <b>D</b> <b>E</b> <b>K</b> <b>A</b> <b>S</b> <b>P</b> <b>D</b> <b>K</b> <b>H</b> <b>R</b> <b>F</b> <b>S</b> <b>L</b> <b>S</b> <b>R</b> <b>Y</b> <b>A</b> <b>K</b> <b>L</b> <b>N</b> <b>R</b> <b>L</b> <b>N</b> <b>P</b> <b>K</b> <b>L</b> <u><b>E</b></u> <b>T</b> <b>F</b> <b>L</b> <b>S</b> <b>K</b> <b>W</b> <b>I</b> <b>G</b> <u><b>D</b></u> <b>R</b> <b>G</b> <b>N</b> <b>R</b> <b>S</b> <b>V</b>	Activity: <i>E. coli</i> = <i>S. aureus</i>
Indolicidin (bovine neutrophils)	I <b>L</b> <b>P</b> <b>W</b> <b>K</b> <b>W</b> <b>P</b> <b>W</b> <b>P</b> <b>W</b> <b>R</b> <b>R</b> -Am	Activity: G+, G-, fungi
Bactenecin (bovine neutrophils)	<b>R</b> <b>L</b> <u><b>C</b></u> <b>R</b> <u><b>I</b></u> <u><b>V</b></u> <u><b>V</b></u> <u><b>I</b></u> <u><b>R</b></u> <u><b>V</b></u> <b>C</b> <b>R</b>	Active against G- but relatively inactive against G+
Tachyplesin 1 (crab)	K <b>W</b> <u><b>C</b></u> <b>F</b> <u><b>R</b></u> <u><b>V</b></u> <u><b>C</b></u> <b>Y</b> <u><b>R</b></u> <u><b>G</b></u> <u><b>I</b></u> <u><b>C</b></u> <b>Y</b> <u><b>R</b></u> <u><b>R</b></u> <b>C</b> <b>R</b> -Am	Activity: G+, G-
Protegrin 1 (porcine leukocytes)	<b>R</b> <b>G</b> <b>G</b> <b>R</b> <b>L</b> <u><b>C</b></u> <b>Y</b> <u><b>C</b></u> <b>R</b> <u><b>R</b></u> <u><b>R</b></u> <u><b>F</b></u> <u><b>C</b></u> <b>V</b> <u><b>C</b></u> <b>V</b> <u><b>G</b></u> <u><b>R</b></u>	Activity: G+, G-
α-defensin HNP-1 Human	A <b>C</b> <b>Y</b> <b>C</b> <b>R</b> <u><b>I</b></u> <u><b>P</b></u> <u><b>A</b></u> <b>C</b> <b>I</b> <u><b>A</b></u> <u><b>G</b></u> <u><b>E</b></u> <u><b>R</b></u> <u><b>R</b></u> <u><b>Y</b></u> <u><b>G</b></u> <u><b>T</b></u> <b>C</b> <b>I</b> <u><b>Y</b></u> <u><b>Q</b></u> <u><b>G</b></u> <u><b>R</b></u> <u><b>L</b></u> <u><b>W</b></u> <u><b>A</b></u> <u><b>F</b></u> <b>C</b> <b>C</b>	Activity: broad spectrum, G+, G-, but greater against G+, fungi
NP-1 rabbit	V <b>V</b> <u><b>C</b></u> <b>A</b> <u><b>C</b></u> <u><b>R</b></u> <u><b>R</b></u> <u><b>A</b></u> <u><b>L</b></u> <u><b>C</b></u> <b>L</b> <u><b>P</b></u> <u><b>R</b></u> <u><b>E</b></u> <u><b>R</b></u> <u><b>R</b></u> <u><b>A</b></u> <u><b>G</b></u> <u><b>F</b></u> <u><b>C</b></u> <b>R</b> <u><b>I</b></u> <u><b>R</b></u> <u><b>G</b></u> <u><b>R</b></u> <u><b>I</b></u> <u><b>H</b></u> <u><b>P</b></u> <u><b>L</b></u> <b>C</b> <b>C</b> <b>R</b> <b>R</b>	Activity: Similar to HNP-1, but more potent
β-defensin BNBD-12 bovine	G <b>P</b> <b>L</b> <b>S</b> <b>C</b> <b>G</b> <u><b>R</b></u> <u><b>N</b></u> <u><b>G</b></u> <u><b>G</b></u> <u><b>V</b></u> <b>C</b> <b>I</b> <u><b>P</b></u> <u><b>I</b></u> <u><b>R</b></u> <u><b>C</b></u> <b>P</b> <u><b>V</b></u> <u><b>P</b></u> <u><b>M</b></u> <u><b>R</b></u> <u><b>Q</b></u> <u><b>I</b></u> <u><b>G</b></u> <u><b>T</b></u> <u><b>C</b></u> <b>F</b> <u><b>G</b></u> <u><b>R</b></u> <u><b>P</b></u> <u><b>V</b></u> <u><b>K</b></u> <b>C</b> <b>C</b> <b>R</b> <b>S</b> <b>W</b>	Activity: G+, G-, fungi
Insect defensin A fungi	A <b>T</b> <b>C</b> <u><b>D</b></u> <u><b>L</b></u> <u><b>L</b></u> <u><b>S</b></u> <u><b>G</b></u> <u><b>T</b></u> <u><b>G</b></u> <u><b>I</b></u> <u><b>N</b></u> <u><b>H</b></u> <u><b>S</b></u> <b>A</b> <b>C</b> <b>A</b> <u><b>A</b></u> <u><b>H</b></u> <b>C</b> <u><b>L</b></u> <u><b>L</b></u> <u><b>R</b></u> <u><b>G</b></u> <u><b>N</b></u> <u><b>R</b></u> <u><b>G</b></u> <u><b>G</b></u> <u><b>Y</b></u> <b>C</b> <b>N</b> <u><b>G</b></u> <u><b>K</b></u> <u><b>G</b></u> <u><b>V</b></u> <b>C</b> <b>V</b> <u><b>C</b></u> <b>R</b> <b>N</b>	Activity: G+, G-
Plant defensin Rs AFP 1	<b>Q</b> <b>K</b> <b>L</b> <u><b>C</b></u> <b>E</b> <u><b>R</b></u> <u><b>P</b></u> <u><b>S</b></u> <u><b>G</b></u> <u><b>T</b></u> <u><b>W</b></u> <u><b>S</b></u> <u><b>G</b></u> <u><b>V</b></u> <b>C</b> <b>G</b> <u><b>N</b></u> <u><b>N</b></u> <u><b>N</b></u> <b>A</b> <b>C</b> <b>K</b> <u><b>N</b></u> <u><b>Q</b></u> <b>C</b> <b>I</b> <u><b>N</b></u> <u><b>L</b></u> <u><b>E</b></u> <u><b>K</b></u> <u><b>A</b></u> <u><b>R</b></u> <u><b>H</b></u> <u><b>G</b></u> <u><b>S</b></u> <b>C</b> <b>N</b> <u><b>Y</b></u> <u><b>V</b></u> <u><b>F</b></u> <u><b>P</b></u> <u><b>A</b></u> <u><b>H</b></u> <u><b>K</b></u> <b>C</b> <b>I</b> <u><b>C</b></u> <b>Y</b> <u><b>F</b></u> <u><b>P</b></u> <b>C</b>	Activity: fungi

<sup>a</sup> G+, G-, Gram-positive and -negative microorganism; Am, amide. Cationic residues are in bold, anionic residues are underlined.

[29,30], also possess antibacterial activity. An examination of the biological activity profiles of these peptides indicates that they are highly variable and not easy to rationalize on the basis of their length, charge or the presence/absence of S–S bridges. Even among peptides in a particular class like cecropins, the activities vary. Rabbit defensins are considerably more potent than human defensins. The minimal inhibitory concentration (MIC) or potencies of the various peptides are not compared as the assays have been performed by different methods and on different strains of microorganisms.

Host-defense antimicrobial peptides have been the subject of extensive structural investigations by a variety of techniques. Circular dichroism (CD) has been widely used to study their conformations in aqueous medium, structure-promoting solvents and lipid vesicles. These studies indicate that the linear peptides like cecropins [31–38], magainins (reviewed in [27]) and other frog skin peptides [20,39] are unordered in aqueous medium and adopt helical structures in structure-promoting solvents like hexafluoroisopropanol and trifluoroethanol (TFE), and in the presence of lipid vesicles. In several peptides, the helical structure is more pronounced in the presence of anionic lipids such as phosphatidylglycerol (PG) and phosphatidylserine (PS). The technique of CD, though a convenient and quick method to assess peptide conformation [40–42], has limitations. For example, it is not possible to determine the conformation at single residue or segment level. It is also not possible to determine accurately the extent of helix,  $\beta$ -sheet and  $\beta$ -turn structures, if they co-exist, although several methods to deconvolute CD spectra have been reported [43–46]. Notwithstanding these limitations, this technique has been extensively used, especially to compare structures of variants and in lipid environment. A characteristic structural feature in the linear antimicrobial peptides is that the helix is markedly amphiphilic in nature.

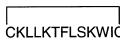
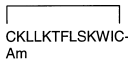
Although nuclear magnetic resonance (NMR) methods can give information about structure at single residue/segment level [47], it has not been employed extensively in studying the conformations of host-defense antibacterial peptides. NMR studies have indicated that cecropin A adopts a helix–bend–helix conformation in the presence of aqueous hexafluoroisopropanol [48]. However, Cecropin P1, a

variant of cecropin A, is a continuous helix [49]. Magainin 2 also adopts helical conformation in TFE [50]. Solid-state NMR data based on  $^{13}\text{C}$  and  $^{15}\text{N}$  chemical shifts in selectively isotope-enriched peptides suggest that magainin occurs in helical and  $\beta$ -sheet structure in the presence of lipid vesicles [51,52]. Since chemical shifts are a reliable index of conformation, it is likely that a fraction of magainin molecules do indeed exist in the  $\beta$ -conformation. Several recent NMR studies on peptides approximately 20–40 residues in length indicate that short peptides do assume multiple conformations [53–57]. Hence, it is conceivable that the linear antibacterial peptides can indeed adopt both helix and  $\beta$ -conformation and this may reflect on their widely differing activities when charges and hydrophobicities are comparable. The solution structures of rabbit defensin NP-2, and NP-5 and human defensin HNP-1 have been determined by NMR [58–62]. The structure of one  $\beta$ -defensin, BNBD-12, has also been determined by NMR [63]. It is evident that both the defensins, in spite of different S–S connectivities, have very similar structures, especially in the  $\beta$ -strand region in a  $\beta$ -hairpin conformation. One important feature that has emerged from NMR studies is that human defensins exist in solution as dimers whereas the rabbit peptides exist as monomers. BNBD-12 also appears to exist as a monomer. Detailed NMR studies on insect and plant defensins indicate that the structure of  $\beta$ -sheet is conserved, especially at the C-terminal region, irrespective of the S–S linkage pattern. However, both insect and plant defensins have a helical segment at the N-terminal region [22,64–66]. Tachyplesin, composed of two S–S bridges, forms a rigid anti-parallel  $\beta$ -structure [67]. The X-ray structure of only one host defense peptide with S–S bridges, HNP-3, has been determined and the structure reveals a  $\beta$ -sheet conformation [68].

Other methods like Fourier transform infrared spectroscopy (FTIR), attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), Raman spectroscopy and oriented circular dichroism (OCD) spectroscopy have been used to a limited extent to investigate the conformational propensities of magainin, cecropin P1 and protegrin 1 [69–72]. FTIR studies also suggest that a fraction of magainin molecules exist in a  $\beta$ -conformation. ATR-

Table 2

Effect of modulating charged, polar and hydrophobic residues on the activities of the 13-residue segment of SPLN, PKLLETFLSKWIG<sup>a</sup>

Peptide	Sequence	Size (charge)	Nature of modification	Effect on activity	Remarks (including structure)
SPF	PKLLETFLSKWIG	13(+1)		Active on <i>E. coli</i> with MIC 50 µg/ml. Hemolytic (100% of 50 µg/ml)	Most hydrophobic segment of SPLN
Substitution analogs of SPF					
S1	PELLKTFLSKWIG	13(+1)	K2 & E5 interchanged	Both antimicrobial and hemolytic activity are lost	
S2	PKLLKTFLSEWIG	13(+1)	E5 & K10 interchanged	-do-	
SPFE	PELLETFLSEWIG	13(-3)	K2 & K10 replaced by E	- do -	Cationic nature essential for activity
SPFK	PKLLKTFLSKWIG	13(+3)	E replaced by K	Increase in positive charge leads to increased antimicrobial activity. No change in hemolytic activity	
SK1	PKLLTLLSKLIG	13(+3)	F & W in SPFK replaced by L	Slightly less active than SPFK	Aromatic residues not critical for activity
SK2	PKLLTKFLSKWIG	13(+3)	T6 & S9 placed at 5 <sup>th</sup> & 10 <sup>th</sup> position	More active than SPFK	Change increases <μH>. Hence more active
SK3	PWLLTKFLSKKIG	13(+3)	Scrambled Sequence of SPFK	Activity completely lost	Change decrease the <μH> and renders it non-surface seeking. Hence loss in activity
PF1	PKLLTKILKSWIG	13(+3)	F7 replaced by (f) in SK2	All analogs are 2-3 fold more active than SPFK	Presence of p-fluo phe leads to increase in antibacterial activity due to better membrane affinity. Hemolytic activity not found
PF2	PKLLTKILKSfIG	13(+3)	F7 and W11 replaced by f		
PF3	PKLLTKFLKSfIG	13(+3)	W11 replaced by f		
Omission and addition analogs of SPFK					
K5	PKLLKFLKKWIG	13(+3)	S & T replaced by Ks	No increase in antibacterial activity over SPFK but hemolytic activity abolished	Increase in positive charge destabilizes helical structure and this may lead to loss of hemolytic activity
WKK	PKLLKTFLSKWKKIG	15(+5)	K12 & K13 added	- do -	- do -
K3S	PKLLKFLSKWIG	12(+3)	T6 omitted	Both antibacterial and hemolytic activity are reduced to half of SPFK	Deletion of either T or S or both of them together does not lead to loss of amphiphilicity and are not critical for activity
K3T	PKLLKTFLKWIG	12(+3)	S9 omitted		
K3	PKLLKFLKWIG	11(+3)	T6 & S9 omitted		
KTF	PKLKTFLSKWIG	12(+3)	L3 omitted	Weakly antibacterial And no hemolytic activity	Removal of L3 alone or with T6 decreases both <H> & <μH> of the peptide and results in loss of surface activity
KF	PKLKFLSKWIG	12(+3)	L3 & T6 omitted		
SL	(Acm)CKLLKTFLSKWIC(acm)	13(+3)	P & G replaced by putative C	Antibacterial activity is five fold less and non hemolytic	-
SAL	(Acm)CKLLKTFLSKWIC(Acm)-Am	13(+4)	P & G replaced by C	Antibacterial activity is two fold lower, non-hemolytic	Amidation of C-terminal leads to increase in positive charge and hence more active
SC		13(+3)	Cyclic analog of SL	No change in antibacterial activity over SL but more hemolytic	
SAC		13(+4)	Cyclic analog of SAL	Four fold decrease in antibacterial activity over SAL but is considerably more hemolytic	Decrease in intrinsic flexibility as compared to linear peptide results in better membrane binding

<sup>a</sup><μH>, hydrophobic moment; <H>, average hydrophobicity; Am, amide.

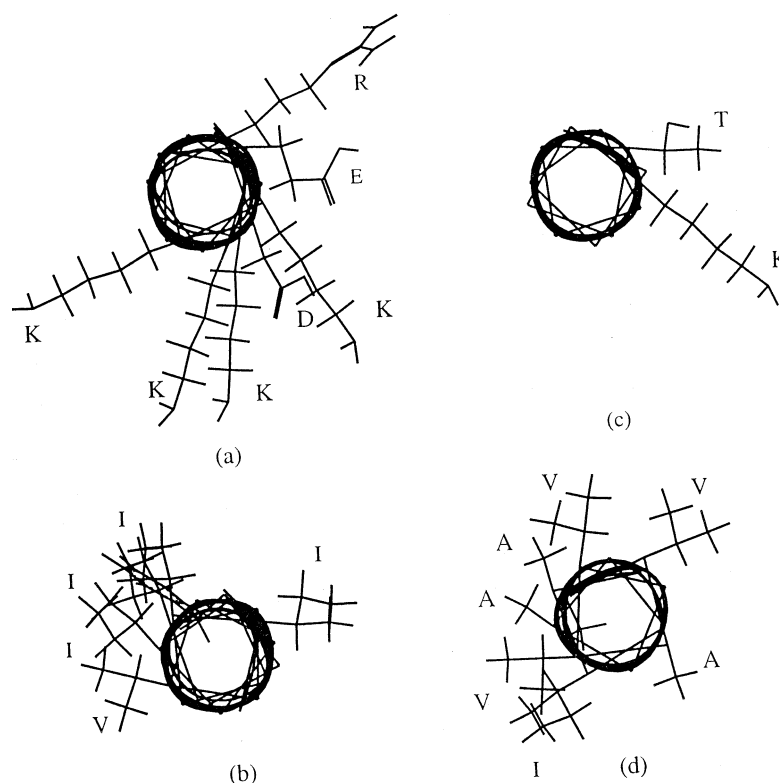


Fig. 1. View of cecropin helices formed by residues 5–21 and 25–37 down the helix axis. (a,b) Position of hydrophilic and hydrophobic side chains of segment 5–21; (c,d) positions of hydrophilic and hydrophobic residues of segment 25–37. Structures were built using Biopolymer module of Biosym.

FTIR and OCD studies have also allowed the determination of the orientation of the peptides at the membrane surface.

The conformations of cecropin A and magainin 2 are shown in Figs. 1 and 2. The structures presented are views through the helix axes. The amphiphilic and hydrophobic nature of the cecropin helices and the amphiphilic nature of the magainin helix are clearly discernible. The structures of the defensins are shown in Fig. 3. The  $\beta$ -sheet structure at the C-terminal region is conserved in all of them. A helical segment preceding the  $\beta$ -sheet region is observed in insect defensin.

### 3. Mechanism of antimicrobial activity

In spite of the considerable variation in primary and secondary structures, net positive charge and distribution of positive charges along the peptide se-

quences, the host-defense antibacterial peptides have a common mechanism of action.

Based on the observation that when the protozoa, *Paramecium cadalum*, *Amoeba proteus* and *Euglena gracilis* were exposed to magainin 2, in pond water or distilled water, swelling of the contractile vacuoles occurred followed by progressive swelling of the organisms, Zasloff proposed that the mechanism of action of magainin could involve membrane perturbation [73]. Zasloff also suggested that the membrane affinity of the peptide could arise as a result of a strongly amphiphilic structure that would be formed if the peptide was helical. Based on conductance experiments in planar bilayers, Duclohier et al. proposed that magainin formed anion-selective channels which were weakly voltage dependent [74]. Cruciani et al. proposed cation specificity for magainin 2 channels with a specificity ratio of 5:1 over anions [75]. The amphiphilic nature of cecropin, and its ability to interact with membranes and form ion-selective

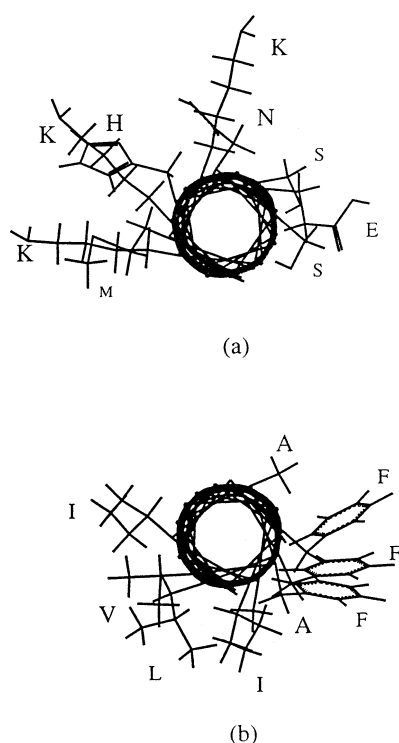


Fig. 2. View of magainin in  $\alpha$ -helical conformation down the helix axis. (a) Positions of hydrophilic residues; (b) positions of hydrophobic residues. Structures were built using Biopolymer module of Biosym.

channels in planar lipid bilayers [76], suggested that cecropins too exert their antibacterial activity by permeabilizing bacterial membranes. The observation of defensin-induced leakage of  $K^+$  and other cellular contents and formation of voltage-dependent ion-permeable channels in planar bilayers [77,78] indicated that defensins also act on bacterial membranes like cecropins and magainins.

The outer membrane (OM) of Gram-negative bacteria has negatively charged lipopolysaccharide (LPS) molecules lining the exterior of the surface [79]. Polycationic molecules bind to the OM and alter the physical structure of the bilayer, which renders it permeable to normally impermeant hydrophobic molecules [80]. The cationic nature of the antibacterial peptides summarized in Table 1 indicates that they could permeabilize the bacterial OM of Gram-negative bacteria by a similar mechanism. Several studies which have monitored bacterial OM permeabilization by cationic peptides support this mode of entry. Binding of magainin 2 to LPS results in dis-

ordering of the fatty acyl chains resulting in structural alterations in the OM-peptidoglycan complexes, as suggested by altered thermotropic properties [81,82]. Magainins also permeabilize LPS-containing liposomes [83]. Divalent cations, which can bind to the anionic sites in the OM, would be expected to inhibit the activity of antimicrobial peptides, if destabilization of the OM is necessary to gain access to the inner membrane (IM) or cytoplasmic membrane. Antimicrobial activity of many peptides including defensins, magainins and seminalplasmin (SPLN) are indeed inhibited by divalent cations [15,83,84]. Permeabilization of the OM by antimicrobial peptides has been monitored by using the hydrophobic fluorescent dye N-1-phenyl naphthylamine (NPN) [85] or assaying the accessibility of the periplasmic enzyme  $\beta$ -lactamase to the normally impermeable cephalosporin substrate PADAC [86]. These molecules are impermeable to the OM and cross the OM barrier only on permeabilization. Using these assays, defensins, indolicidin and bactenecins have been shown to be capable of permeabilizing the bacterial OM [87–92].

The IM of Gram-negative bacteria is composed of anionic lipids like phosphatidyl glycerol (PG) and cardiolipin which would favor the association of cationic peptides. Permeabilization of the IM has been assessed by measuring the influx of a normally impermeable chromogenic substrate to a cytoplasmic enzyme in presence of the antimicrobial agent,  $\beta$ -galactosidase, and its substrate ortho nitrophenyl galactoside (ONPG) [86–92]. The substrate ONPG is normally taken up across the IM with the help of lac permease and in the absence of this protein transporter, no ONPG influx is possible. Enhanced influx of ONPG in the presence of an antimicrobial agent would reflect permeabilization of the IM. Using an assay where permeabilization of both the OM and IM can be concurrently measured [86], Lehrer et al. demonstrated that in the case of human defensin HNP-1, OM permeabilization was closely followed by IM permeabilization and the latter event was associated with loss of viability of cells due to cessation of respiration [88]. IM permeabilization has also been assayed by monitoring the fluorescence of the membrane-potential sensitive cyanine dye diS-C<sub>3</sub>-(5) [92]. The fact that the cytoplasmic membrane may indeed be the target of action of antimicrobial pep-

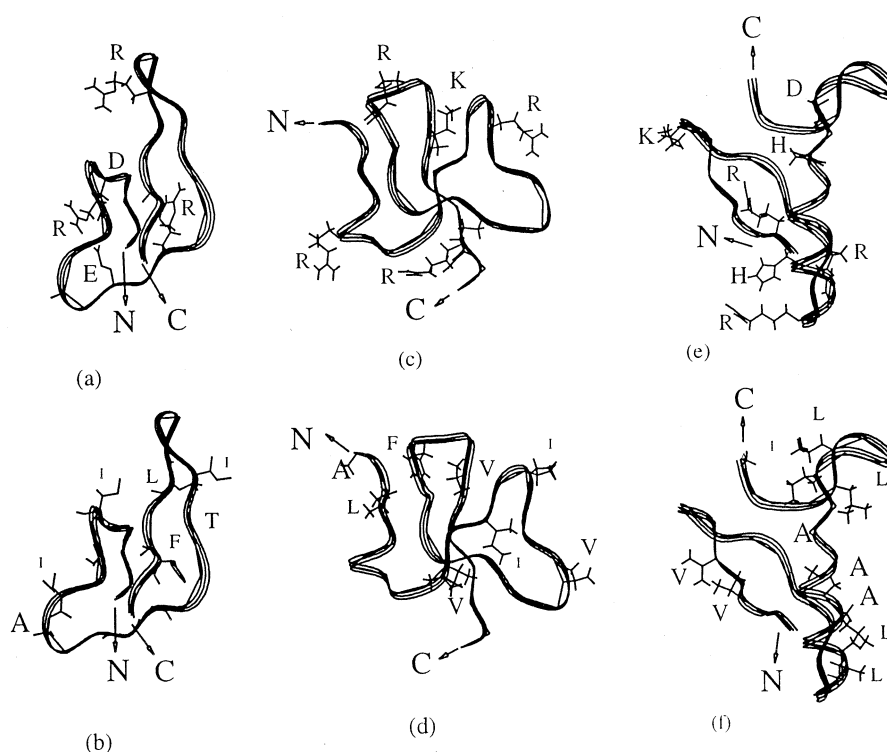


Fig. 3. Structures of  $\alpha$ ,  $\beta$  and insect defensin. (a,b) Hydrophilic and hydrophobic residues of  $\alpha$ -defensin (HNP-3); (c,d) hydrophilic and hydrophobic residues of  $\beta$ -defensin BNBD-12; (e,f) insect defensin A. Structures were generated from co-ordinates obtained from Protein Data Bank.

tides was established in a study on insect defensin A, which effectively depolarized the cytoplasmic membrane of *Micrococcus luteus* besides causing leakage of cytoplasmic potassium and ATP [78]. SPLN was also capable of effectively permeabilizing the cytoplasmic membrane of *Escherichia coli* cells to ONPG, indicating that SPLN could provide an additional pathway for ONPG influx by permeabilizing the IM [84]. Cecropins have the ability to induce a hyperosmotic stress response in *E. coli* [93]. The authors have proposed that cecropin in the periplasmic space makes stable contacts between the inner and outer membranes which prevent the shrinkage of the cytoplasmic compartment in response to hyperosmotic water influx.

The morphology of Gram-negative and -positive bacteria treated with cationic antimicrobial peptides as examined by electron microscopy clearly indicates that the cells are killed due to disruption of the cytoplasmic membrane [87,94–98]. Membrane defects were observed even at low concentration at which the peptides were not bactericidal. However, no le-

sions in the bacterial cell surface were discernible. Recent reports provide additional evidence that the initial site of interaction of cationic antibacterial peptides with Gram-negative bacteria is the OM. Investigation of the mechanism by which *Salmonella* induce resistance to cationic antibacterial peptides like PGLa and protegrins has indicated that in resistant strains, there is acylation of lipid A by palmitic acid [99]. This modification is presumed to alter the OM structure so as to prevent its permeabilization by cationic peptides. Lipid A palmitoylation in *E. coli* and *Yersinia enterocolitica* in response to low  $Mg^{2+}$  growth conditions also induces resistance to cationic antibacterial peptides. Analysis of resistance profiles in *Bordetella* species [100] have indicated that *B. pertussis* is much more susceptible to cecropins and magainin 2 as compared to *B. bronchiseptica* and this arises due to the lack of the highly negatively charged O-specific sugar side chains on the OM.

Peptides like magainins, cecropins and defensins exert their effects on the bacterial cytoplasmic membrane so rapidly that it is not easy to determine



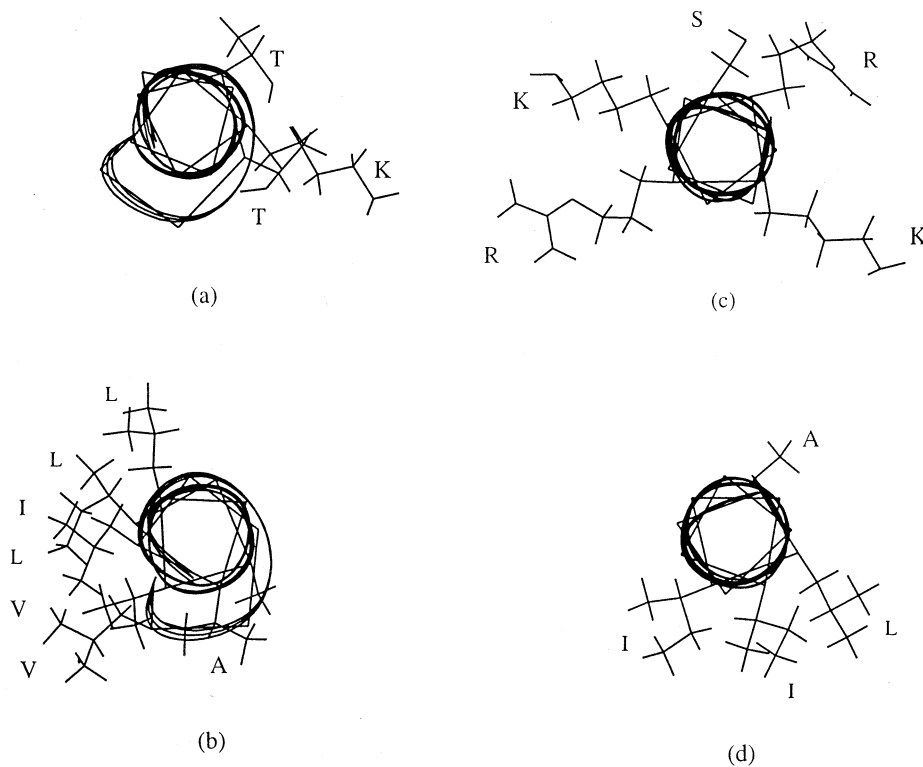


Fig. 4. View of melittin helix down the helix axis. (a,b) Position of hydrophilic and hydrophobic residues in helical segment 1–13; (c,d) positions of hydrophilic and hydrophobic residues formed by helical segment 15–26.

whether there are other intracellular targets for these peptides. In a recent study, it has been demonstrated that pretreatment of *Staphylococcus aureus* with novobiocin, an inhibitor of bacterial DNA gyrase subunit B, or with 50S ribosomal subunit protein synthesis inhibitors such as daltopristin, blocked killing by defensin HNP-1 [101]. However, killing of bacteria by multiple mechanisms remains to be established unequivocally. The finding that all D-analogs of cecropins and magainin [102,103] also exhibit antimicrobial activities identical to that of their natural L-analogs have effectively ruled out the involvement of chiral recognition.

In vivo experiments in mice suggest that the antibacterial activity of neutrophil defensins involves recruitment of macrophages, granulocytes and lymphocytes at the site of infection [104]. Defensins also appear to enhance systemic IgG involving  $CD_4^+$  Th-1 and Th-2 type cytokines, suggesting that innate immunity is linked to adaptive immune system [105].

#### 4. Identification of active peptide segments from antibacterial peptides

Detailed structure–function studies on cecropins and magainins have highlighted the requirement for helical structure in the presence of lipid vesicles and appropriately positioned cationic residues for activity [23,24]. Extensive studies have also delineated regions of endogenous antibacterial and hemolytic peptides like SPLN, dermaseptin, pardaxin and melittin that are shorter than the parent peptides but possess antibacterial and/or hemolytic properties. Hybrids of cecropins and melittin have also been generated with antibacterial activities. Hemolytic peptides like melittin and pardaxin have been engineered to generate molecules with only antibacterial activity. These studies have revealed that short peptides composed of 11–15 residues can indeed permeabilize membranes and the mechanisms of action on bacteria are similar to that of the longer peptides. These aspects are reviewed in this section.

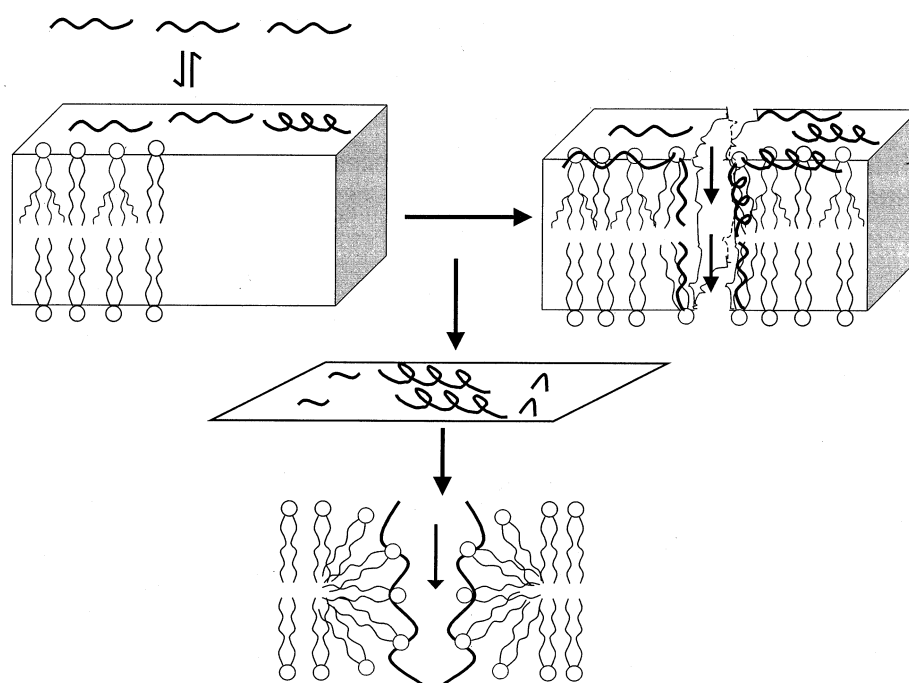


Fig. 5. Model depicting permeabilization of bacterial membranes by peptides. Thick lines correspond to peptides. Binding of cationic peptides to the bacterial cell surface, which is negatively charged, results in membrane destabilization which allows entry of peptides to the plasma membrane. The peptides bind to the membrane surface and reorient to form pores which results in membrane permeabilization. The structure of the toroidal pore shown in the figure has been proposed by Matsuzaki and co-workers [27] and Ludtke et al. [201].

#### 4.1. Seminalplasmin (SPLN)

SPLN, a 47-residue protein isolated from bovine seminal plasma (sequence indicated in Table 1), has broad-spectrum activity against Gram-negative and -positive bacteria as well as yeast without any hemolytic activity [106–108]. The protein was observed to inhibit ribosomal RNA synthesis in *E. coli* and other target microorganisms [109,110]. SPLN also had the ability to rapidly permeabilize the outer and cytoplasmic membranes of *E. coli*, suggesting that the protein could exert its activity by permeabilizing bacterial membranes [84]. The protein was also capable of lysing dividing mammalian cells but not resting cells [111]. Chou–Fasman analysis for the secondary structure of SPLN predicted two possible  $\alpha$ -helical regions between 8–22 and 28–39 along with two  $\beta$ -turns at residues 6–9 and 41–44. The Garnier–Robson method of analysis suggested  $\alpha$ -helical segments for residues 1–10, 13–25 and 27–35 [112]. The 28–40 segment of SPLN also corresponded to its most hydrophobic stretch when analyzed by the method of

Kyte and Dolittle [112]. Helical wheel projections indicated two amphiphilic  $\alpha$ -helical regions with clearly demarcated hydrophobic and hydrophilic faces. Hydrophobic moment analysis according to the Eisenberg method assigned these helical segments to the ‘surface seeking’ region. SPLN also is known to bind strongly to calmodulin in a  $\text{Ca}^{2+}$ -dependent manner and functions in vitro as an antagonist [113]. Such interactions are characteristic of cationic amphiphilic helical peptides. Segment 13–25 showed considerable homology to the calmodulin-interacting segments of other proteins/peptides [114]. NMR studies have also indicated that SPLN forms a more compact structure in the presence of detergent micelles with an  $\alpha$ -helical conformation for residues of 21–27, 31–34 and 35 to C-terminus [115].

Synthetic peptides corresponding to the putative  $\alpha$ -helical segments 14–26 and 28–40, as well as the 27-residue fragment (P27) encompassing both these segments, were synthesized and evaluated for biological activities [116–118]. These peptides, with the sequences SLSRYAKLANRLA (SLS), PKLLET-

FLSKWIG (SPF) and SLSRYAKLANRLANPKL-LETFLSKWIG (P27), respectively, exhibited differential antimicrobial activity. SLS and SPF exhibited activity only against *E. coli* with MICs of 60 µg/ml and 50 µg/ml, respectively, compared to 10–20 µg/ml of SPLN. Unlike SPLN they were inactive on Gram-positive microorganisms. Thus, in both spectrum as well as potency, the shorter synthetic peptides of SPLN were less active than the parent peptide. P27, on the other hand, exhibited antibacterial activity against Gram-negative and -positive bacteria with MICs comparable to those of SPLN [116]. The SLS peptide had no hemolytic activity, whereas SPF had considerable hemolytic activity. P27 possessed hemolytic activity but it was less pronounced compared to its antimicrobial activity. SPF and P27 could rapidly permeabilize the plasma membrane of *E. coli* just like SPLN [117,118]. While it is possible that regions other than SPF or the stretch corresponding to the P27 peptide in SPLN could be responsible for the lack of hemolytic activity and the selective activity on dividing eukaryotic cells, it is clear that the segment corresponding to SPF is primarily responsible for the membrane-perturbing activity of SPLN. The interactions of SPLN and SPF with model membranes have been investigated [119]. SPLN bound more strongly to PG vesicles as compared to PC vesicles. Localization studies indicated that the tryptophan residue in both the peptides was located away from the lipid head-group region. Both the peptides could cause the release of entrapped carboxy fluorescein (CF) from lipid vesicles. Analysis of the CF release data indicated a first-order dependence on peptide concentration, suggesting that the peptides did not aggregate. CD studies indicated that SPF and P27 adopted helical conformation in hydrophobic environment [118]. The spectra indicated that while P27 had a helical content comparable to SPLN, that of SPF was lower. Synthetic peptides corresponding to the N-terminal segment (1–15 residues) of SPLN did not exhibit either antimicrobial or hemolytic activities (unpublished results). Hence, an approach based on secondary structure analysis and hydrophobicity does appear to help in identifying short peptides possessing biological activity.

#### 4.1.1. Analogs of SPF

As the 13-residue peptide SPF corresponding to

the most hydrophobic segment of SPLN inhibited the growth of *E. coli* and exhibited hemolytic activity, it appeared to be an ideal molecule for studying the various requirements for biological activity. Hence, several analogs of SPF were synthesized [120–124], with an aim of understanding how the overall charge and position of charges would modulate its biological activity. The primary structures of the various analogs, nature of modifications and effects on activity are summarized in Table 2. The arrangement of charged residues in SPF, i.e., KXXEXXXK, appeared to be important for its activity, as interchanging of charged residues (analogs S1 and S2) resulted in complete loss of activity [120]. Although peptides S1 and S2 were inactive, S1 had a helical content comparable to SPF, whereas S2 was completely unordered. The inability of S2 to acquire helical structure could be the reason for its inactivity. Presence of E near the C-terminal end appeared to destabilize the helix. In the case of S1, a possible salt bridge between E and K at positions i and i+3, could stabilize the helical structure. Such salt bridge formation is likely to be unfavorable for membrane interaction. SPFE and SPFK are analogs of SPF in which charged residues have been replaced. SPF has 2 Ks and 1 E and carries a net charge of +1 at pH 7.0. In SPFE both Ks of SPF were replaced by Es and in SPFK the single E was replaced by K [120]. SPFE was completely devoid of both antimicrobial and hemolytic activities. Considering that a negatively charged peptide cannot interact with anionic biological membranes, this result would not be surprising. On the other hand, SPFK, with a net positive charge of +3, exhibited fivefold higher antimicrobial activity compared to its parent peptide SPF, without any discernible change in its hemolytic activity [121]. SPFK also had a much broader spectrum of antibacterial activity than SPF. Enhanced antimicrobial activity of SPFK stems primarily from the increased net positive charge on the peptide, as this modification did not bring about significant changes in hydrophobicity, hydrophobic moment or conformation. These analogs highlighted the importance of positive charge on the peptide for its antimicrobial activity. The analog SK-1, in which both the aromatic amino acids of SPF, F and W, were replaced by Ls, was completely non-hemolytic, while retaining antimicrobial activity. This suggested that, while ar-

omatic amino acids may play a role in the interaction of the peptides with eukaryotic membranes, they are not critical for antimicrobial activity [120]. The other two analogs, SK2 and SK3, were designed to check the importance of the hydrophobic moment for biological activity. In these two analogs the sequence of SPFK was scrambled in such a way that in SK-2 there was an increase in hydrophobic moment and in SK-3 the hydrophobic moment was lower. SK-2 exhibited marginally increased antimicrobial activity without any hemolytic activity and SK-3, as expected, was devoid of both the activities. The peptides that exhibited antimicrobial activity could also permeabilize lipid vesicles composed of PE and PG. Permeabilization was determined by monitoring the release of entrapped CF.

#### 4.1.2. Addition and omission analogs of SPFK

Deletion or addition of amino acids in the middle of the sequence of amphiphilic  $\alpha$ -helical peptides can change the relative distribution of polar and apolar amino acids and thereby their hydrophobic moments. Hence, the consequences of selective addition and deletion of polar and apolar amino acid residues were investigated [122]. Several variants of SPFK were generated by: (i) substituting S and T residues by Ks; (ii) deletion of S and T residues individually and together; (iii) by adding extra Ks on the polar face of the helix; and (iv) deleting a L along with or without T. The analogs generated, together with their activities, are listed in Table 2. Addition of 2 Ks or replacement of S and T with Ks leads to an increase in positive charge and was expected to enhance the antimicrobial activity. However, no increase in activity was observed. These peptides with increased positive charge also exhibited decreased helical content, possibly due to electrostatic repulsion between the positively charged lysine side chains. Deletion of S and T individually or together did not have much effect on antimicrobial activity, as such deletions do not result in decrease in hydrophobic moment. On the other hand deletion of L3 alone or along with T resulted in considerable decreases in hydrophobic moment and average hydrophobicity and consequently loss of biological activity.

#### 4.1.3. Cyclic analogs of SPFK

Disulfide bridges are known to play an important

role in the stabilization of secondary structure as well as activity in a large number of antimicrobial peptides. In order to understand the effect of introducing a disulfide bridge in a linear antimicrobial peptide, analogs of SPFK were synthesized with cysteine residues at the N- and C-terminal ends in place of the existing amino acids [123]. While one analog had the acid at the C-terminus, the other analog was amidated. Activities of the linear analogs in which cysteines were protected with Acm groups and the cyclized analogs after deprotection were assessed. Replacement of the terminal residues with cysteines resulted in a decrease of both antimicrobial and hemolytic activities. However, amidation of the C-terminal carboxyl group resulted in considerable enhancement in antimicrobial activity without any hemolytic activity. Cyclization of these analogs showed dramatic increases in their hemolytic activities. The cyclic analogs associated more strongly with lipid vesicles than the linear peptides and were much more effective in causing lysis of erythrocytes.

#### 4.1.4. Introduction of *p*-fluorophenylalanine (*f*) into PKLLTKFLKSWIG

The enhanced antibacterial activity of fluoroquinolones appears to arise due to increased hydrophobicity of the molecules resulting in more effective permeation of bacterial membranes. Hence, the effect of introducing *p*-fluorophenylalanine on antibacterial activity was examined in the 13-residue peptide PKLLTKFLKSWIG [124]. The sequences as well as their activities are summarized in Table 2. Introduction of these amino acids enhanced the antimicrobial activity of the peptide. Activity was also observed against *Candida utilis* which was not seen with the parent peptide. Although one analog showed hemolytic activity, the concentration for 50% hemolysis was well above the MIC. Hence, *p*-fluorophenylalanine appears to be a good candidate for incorporation into a peptide in order to obtain selective broad-spectrum antimicrobial activity.

In summary, studies on the variants of SPF indicate that addition of cationic amino acids and/or deletion of polar amino acids such as S and T can modulate the biological activities of short peptides composed of  $\sim 13$  residues. Those predicted to be surface-active on the basis of hydrophobic moment and average hydrophobicity values have antibacterial

activity. However, hemolytic activity was observed only for SPF and SPFK. Peptides possessing hemolytic and antibacterial activities were most effective in permeabilizing lipid vesicles as compared to peptides having only antibacterial activity. Also, peptides showing selective antibacterial activity had lower propensity for helical conformation in a hydrophobic environment.

#### 4.2. Pardaxin

Pardaxin is an ichthyotoxic peptide secreted by sole fish of the genus *Pardachirus* and displays cytolytic activity. The structural and charge requirements for membrane and cytolytic activities in the toxin, which has the sequence GFFALIPKII<sup>10</sup> SSP-LFKTLLS<sup>20</sup> AVGSALSSS<sup>30</sup> GEQE, have been extensively investigated [30]. The peptide also exhibits broad-spectrum antibacterial activity [95]. Modification of E residues resulting in increase of positive charge increased antibacterial potency and enhanced hemolytic activity. Although deletion of the C-terminal 10 residues resulted in a dramatic decrease in antibacterial potency, addition of NH(CH<sub>2</sub>)<sub>2</sub>-NH<sub>2</sub> at the C-terminal of the shortened peptide restored antibacterial activity even when the K residues were deleted. Peptides where the N-terminal 10 residues or the C terminal 20 residues were deleted were inactive even when NH-(CH<sub>2</sub>)NH<sub>2</sub> was present at the C terminus [95]. Substitution of P7 by A in pardaxin resulted in enhanced hemolytic activity but decreased antibacterial activity [125]. The N-terminal 1–18 segment had threefold lower activity against *E. coli* as compared to pardaxin but had no hemolytic activity [125]. When P7 was replaced by A in this peptide, antibacterial activity was unaffected though a weak hemolytic activity was observed [126]. Introduction of D amino acids to create diastereomers reduced the hemolytic activity considerably whereas the antibacterial activities were retained [96].

The conformations of the peptides were examined by CD spectroscopy in aqueous TFE [95,96,125]. The helical content, as determined by  $\theta_{222}$  values, did not directly correlate with activity. Some of the inactive peptides did have appreciable helical content. The diastereomeric peptides did have lower helical content than pardaxin and also had lower hemolytic activity. Permeabilization of model membranes

composed of PC appeared to correlate with hemolytic potency. A peptide derived from the N-terminal 1–18 segment of pardaxin, GFFALIAQIISSPL-FQTL-Am, where the K residues were replaced by Q, had hemolytic activity but was devoid of antibacterial activity [126]. The peptide segment GFFALIP-KIISSPLFK-Am, which did not have any biological activity, could be engineered by judicious positioning of K and P residues to generate peptides with antibacterial and/or hemolytic activity [127].

In summary, studies on pardaxin and its analogs indicate that a net cationic charge of +1 or +2 is sufficient for antibacterial and hemolytic activities, provided other features in the peptide favor pore formation in membranes. Lower helical content specifically favors specific antibacterial activity as hemolytic activity is considerably decreased, probably due to inefficient pore formation. The structural and charge requirements for biological activity correlate with model membrane permeabilization of zwitterionic and anionic vesicles. It appears that in peptides that have strong pore-forming abilities, which of course leads to antibacterial and hemolytic activities, modulation of structure, especially introducing structural flexibility in the molecule thereby reducing effective pore formation, results in loss of hemolytic activity but not antibacterial activity.

#### 4.3. Dermaseptin

Dermaseptin is a 34-residue linear peptide having the sequence ALWKTMLKKL<sup>10</sup> GTMALHAGKA<sup>20</sup> ALGAAADTIS<sup>30</sup> QGTQ. It has been isolated from the skin extract of the frog *Phyllomedusa sauvagii* and is active against pathogenic fungi and bacteria at micromolar concentrations [128,129]. The activities of several truncated analogs of the antibacterial peptide have been examined. While the analogs composed of amino acids 14–34, 16–34, 20–34, 28–34, 1–4, 1–8, 10–19 had no activity, the peptide corresponding to residues 1–18 had activity comparable to that of the parent peptide [130]. CD studies indicated an  $\alpha$ -helical conformation in TFE. The inactive 16–34 peptide was unordered even in TFE. The N-terminal segment of another member of the dermaseptin family, i.e., ALWKNMLKGIG-KLAGK-Am, also showed potent antimicrobial activity [131]. Thus, the N-terminal segment is a major

determinant of structure and biological activity of dermaseptin.

#### 4.4. Melittin

Melittin, a 26-residue hemolytic peptide which is a major component of the venom of European honey bee *Apis mellifera*, has been the subject of extensive investigations directed towards understanding the molecular mechanism of hemolysis [29]. There have been numerous studies on the interaction of melittin with lipids with a view to understand the various facets of the interaction of membrane proteins with membranes [29]. The broad-spectrum antibacterial activity of melittin was observed in 1968 [132] but structural requirements for antibacterial activity has been addressed only in recent years. All D-melittin has hemolytic and antibacterial activities comparable to the L-enantiomer, indicating the absence of chiral recognition in the manifestation of its biological activities [102].

The conformation of melittin (GIGAVLKVL-TTGLPALISWIKRKRQQ-Am), as examined by X-ray crystallography, indicates a helix–bend–helix [133] structure with the bend region positioned between residues 11–15. Lines drawn through the helix axes of residues 1–10 and 16–26 intersect with an angle of  $\sim 120^\circ$ . The N-terminal segment of 20-residues is highly hydrophobic with only one charged residue K at position 7. In contrast, the C-terminal 6 residue segment is highly cationic with two Ks, two Rs, two Qs with the C-terminal also amidated. The N-terminal helical segment is amphiphilic in nature as shown in Fig. 4. Structure–function studies indicate that replacement of P14 with A removes the bend in the structure. A14 melittin was 2.5-fold more hemolytic than its parent peptide, but it formed less stable voltage-dependent channels, thereby indicating that channel formation may not dictate its hemolytic activity [29]. The amphiphilic helical segment of melittin between residues 1–20 appears to play only a structural role in its activity, as the actual sequence could be replaced with a sequence capable of forming amphiphilic  $\alpha$ -helical structure, but having minimum homology to the native segment, without any loss in its activity. The segment 1–20, although membrane active, does not possess any lytic activity. The cationic segment 20–26 was also inactive [134,135].

Analysis of the antibacterial and hemolytic activities of 24 individual omission analogs of melittin indicated that deletion of L(6), K(7), V(8), I(9), I(13), I(16), I(17) and W(19) resulted in a considerable decrease in hemolytic activity [136]. However, deletion of amino acids in the bend region did not affect hemolytic activity. The changes indicated above also resulted in lower antibacterial activity but the decrease in activity as compared to melittin was less pronounced than the loss in hemolytic activity. Interestingly, deletion of individual amino acids in the C-terminal segment had no effect on activity. However, K7 appears to be important for activity. Based on the analysis of retention times during reverse-phase high-performance liquid chromatography, it has been suggested that deletion of amino acids that caused lower hemolytic activity had lower amphiphilicity (decreased amphiphilicity was correlated with early retention times). Hence, the study clearly indicated the requirements for an amphiphilic structure for hemolytic activity. The antimicrobial potencies of the various omission analogs indicated that the requirements of amphiphilic structure for antimicrobial activity was less stringent as compared to hemolytic activity [136].

The K at position 7 in melittin is fully solvent exposed in the tetrameric state. The conformation and aggregation properties, as well as biological activities of single substitution analogs at this position, have been examined [137]. Replacements by I, L or V residues resulted in considerable decreases in antibacterial and hemolytic activities. Analogs in which the replacements were A, D, E or G had slightly lower hemolytic activity as compared to melittin. There was also a decrease in antibacterial potency. All the inactive analogs had lower hydrophobic moments as compared to the active analogs. The active analogs had the ability to form tetramers in the presence of salt, unlike the inactive analogs. Analysis of CD data by curve-fitting methods indicated varying proportions of helix and  $\beta$ -structures in the presence of lipid vesicle (PC/PS). In aqueous TFE, all the peptides had similar helical propensities. Values of the concentration of lipid when 50% peptide was bound did not appear to directly correlate with biological activity. The analog in which was K7 was replaced by W did not appear to aggregate into tetramers, but had hemolytic activities comparable to melittin, although

the antibacterial activity was considerably lower. CD data also indicated the presence of helical and  $\beta$ -sheet structures in the presence of lipid vesicles for this peptide.

The requirement of amphiphilic helical structure for hemolytic activity but not for antimicrobial activity is also borne out by the studies on diastereomeric, retro and retro-enantiomers of melittin [138,139]. Diastereomeric melittin, where V5, V8, I17, and K21 were replaced by D-isomers, showed a very low propensity for helical structure and did not lyse erythrocytes but had antimicrobial activity [139]. The MIC values were only slightly higher than melittin. Retro melittin and retro-enantio melittin, which had a high propensity for  $\beta$ -structure in aqueous medium but had high helical content in the presence of hexafluoroisopropanol, had antimicrobial activity comparable to melittin but considerably lower hemolytic activity. Thus, hemolytic activity appears to be sensitive to direction of amide bonds and helix dipole moment [138].

Studies involving single amino acid omission analogs of melittin revealed a critical role for W19 in its biological activity [136]. Either chemical modification or replacement of W19 with L resulted in a remarkable decrease in activity, thus confirming the importance of this residue [140,141]. A detailed fluorescence study indicates that the W residue is localized just below the head-group region in lipid bilayers [142]. It is conceivable that this orientation is essential for activity. However, the position of W in the sequence does not appear to be very critical for activity, as analogs of melittin containing a single W residue at positions 9, 11 or 17 also exhibited hemolytic activities with potencies in the order  $MLT-W17 > MLT-W19 = MLT-W11 > MLT-W9$  [143]. Melittin forms tetrameric structures at high peptide concentrations or at high salt concentrations, but the other analogs of melittin such as  $MLT-W17$ ,  $MLT-W9$  and  $MLT-W11$  did not form tetramers under these conditions. Hence, the ability to form soluble tetrameric structures appeared to be unrelated to lytic activity.

The segment 1–7 in melittin appears to be very important in modulating its biophysical as well as its biological properties, as the 8–26 peptide did not permeabilize model lipid membranes [144]. Antimicrobial and hemolytic activities of this fragment

are not known. A 15-residue synthetic peptide, corresponding to the C-terminal end of melittin, which encompasses its most amphiphilic segment, exhibited 5–7-fold less antimicrobial activity than melittin [145]. The hemolytic activity of this peptide was 300 times less than that of melittin. An analog of this peptide, in which two cationic residues have been transposed to the N-terminal region from the C-terminal region, had antibacterial activity comparable to that of melittin but considerably lower hemolytic activity. The biological activities of the peptides could be rationalized on the basis of structure and aggregation.

Hybrid peptides composed of various segments of cecropin and melittin have been synthesized and examined for biological activity [102,146,147]. Peptides composed of the amphiphilic 1–10 N-terminal segment of cecropin A and 1–13 region of melittin showed broad-spectrum antimicrobial activity with low hemolytic activity.

## 5. Requirements for activity in naturally occurring short antibacterial peptides

### 5.1. Indolicidin

The 13-residue antibacterial peptide indolicidin, having the primary structure ILPWKWPWWP-WRR-Am, has a mole percentage of tryptophan which is the highest among antibacterial peptides [148]. The peptide has been isolated from bovine neutrophils and belongs to the cathelicidin family of proteins, which have a common proregion and a variable C-terminal antimicrobial domain [149], and is likely to have a role as a host-defense peptide. The unusual amino acid composition has led to studies directed towards delineation of the role of multiple tryptophan residues in its biological activity as well as interactions with model membranes.

#### 5.1.1. Antimicrobial spectrum

Natural indolicidin, amidated at the C-terminal, was found to be bactericidal against *E. coli* and *S. aureus* in phosphate buffer between 10–20  $\mu\text{g/ml}$  concentrations and 25  $\mu\text{g/ml}$  could virtually sterilize  $10^8$  *E. coli* cells in  $\sim 20$  min [148]. Subsequently, synthetic indolicidin, which was identical to natural in-

dolicidin, was shown to exert activity against fungal strains of *Candida albicans*, *C. neoformans*, *Saccharomyces cerevisiae* and *C. utilis* with comparable MICs [150]. Synthetic analogs of indolicidin with C-terminal free acid as well as C-terminal methyl ester have also been shown to possess antimicrobial activity against *E. coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *S. aureus* and *S. epidermidis* with MICs between 4–64  $\mu\text{g/ml}$  [90]. Recently, a study comparing the in vitro activity of indolicidin on 202 clinical isolates of Gram-positive and -negative bacteria has shown that indolicidin is effective on a large number of these isolates [151]. Indolicidin exhibits antiviral activity against HIV-1 [152]. Although indolicidin has impressive and potent antimicrobial activity, it has cytotoxic activity against mammalian cells and exhibits hemolytic activity beyond 15  $\mu\text{M}$  [153]. It also has been shown to possess cytotoxic activity against rat and human T-lymphocytes [152].

#### 5.1.2. Mechanism of action

Indolicidin has been shown to permeabilize the outer membrane of *E. coli* [90,154]. Its C-terminal free acid analog effectively permeabilized the cytoplasmic membrane [90]. Experimental evidence has not been presented for the permeabilization of the cytoplasmic membrane for either natural indolicidin or the C-terminal methyl ester, which closely resembles the natural peptide. Based on conductance measurements with planar bilayer membranes, it has been proposed that indolicidin forms channels in lipid membranes [90]. This suggests that membrane permeabilization may indeed be the basis of its antimicrobial action. However, treatment of microbial cells with indolicidin does not lead to their lysis [90]. Indolicidin did not even render the treated cells prone to lysis by lysozyme [90]. This is in sharp contrast to the action of other well-characterized lytic peptides like cecropins, magainins, defensins and SPLN, which readily lyse the target cells [15–18]. Treatment of *E. coli* cells with indolicidin induced filamentation of cells, a phenomenon observed in the case of antimicrobial agents that block DNA synthesis [155]. Indolicidin also inhibited DNA synthesis in *E. coli* at concentrations at which RNA and protein synthesis were either partially affected or not affected at all. Thus, inhibition of macromolecular synthesis, in addition to membrane permeabilization, appears to be

a plausible mechanism of its antimicrobial action. Hemolytic activity, in contrast, appeared to be directly related to its membrane-associating properties, as an analog with decreased affinity for lipid vesicles was non-hemolytic, while its antimicrobial activity was unaffected [156].

Hydrophobic amino acids constitute  $\sim 54\%$  of indolicidin with five Ws and one each of I and L residues. It also has three residues of P, an amino acid which also is implicated in the assembly of membrane proteins. Fluorescence spectroscopy and equilibrium dialysis measurements have indicated that indolicidin binds strongly, though reversibly, to lipid vesicles composed of zwitterionic and anionic lipids with free energies of transfer of  $-8.8$  and  $-11.5$  kcal/mol, respectively [157]. Based on W fluorescence data, location at the bilayer interface has been suggested for indolicidin [156]. CD studies indicate that the peptide was unordered in aqueous medium and was more ordered in a micellar environment [90,154,157]. It appears that the peptide does not adopt an helical conformation. Hence, in this tryptophan-rich peptide, binding to model membranes as well as antibacterial activity are observed even in the absence of ordered secondary structure.

Several analogs have been synthesized in order to delineate the charge requirements and also to understand the importance of W and P residues for activity. Indolicidin is amidated at the C-terminal end. A synthetic analog with C-terminal free acid (Ind-OH) also exhibited antimicrobial activity, but an analog with C-terminal methyl ester (Ind-OMe), which resembles native peptide in its charge, was considerably more active than Ind-OH [90]. An analog of indolicidin lacking the C-terminal R13 amide of indolicidin had much less antimicrobial and anti-fungal activity as compared to indolicidin [152]. Introduction of K residues in indolicidin, i.e., ILKKWPWWPWRK and its C-terminal methyl ester, had fourfold greater potency than indolicidin acid but these peptides were non-hemolytic [91]. These observations reflect the importance of a net positive charge for antibacterial activity. Replacement of all the W residues with F residues had no significant effect on its antimicrobial activity, but almost completely abolished its hemolytic activity [154]. Tryptophan residues can promote partitioning of the peptide into the lipid bilayer [5] and in turn appear to play a major role in the he-



molytic activity of indolicidin. These attributes do not seem to be vital for its antimicrobial activity. Other analogs of indolicidin, in which W4, W6 and W8 were replaced by I or G residues, exhibited enhanced antimicrobial activity as compared to indolicidin, again confirming the above observation [158]. However, the hemolytic activity of these analogs has not been reported. The three P residues of indolicidin also do not appear to be vital for its biological activity. An analog in which all these P residues were replaced by A had undiminished antimicrobial activity, but increased hemolytic activity [154].

### 5.2. Other tryptophan-rich antimicrobial peptides

A 13-residue synthetic peptide corresponding to the tryptophan-rich region of a putative cathelicidin obtained from the cDNA library of porcine bone marrow had antimicrobial activity against both Gram-positive and -negative bacteria [159]. It also had weak activity against *A. fumigatus*. A family of lipid-interacting proteins with potential antimicrobial function, named puroindoline a and b, have recently been characterized from wheat endosperm [160]. Interestingly, these proteins contain tryptophan-rich amphiphilic domains which are important for their physico-chemical as well as biological properties. Interaction of puroindoline a with model lipid membranes has recently been reported and it has been found to interact with phosphatidylglycerol vesicles with high affinity, leading to disruption of acyl chain packing. However its interaction with phosphatidylcholine appears to be weak. Although the mechanism of action is not clear, tryptophan-rich peptides do appear to possess broad spectrum antibacterial activity.

## 6. Structure–function relationship studies in antimicrobial peptides containing one or more disulfide bridges

### 6.1. Peptides containing a single disulfide bridge

#### 6.1.1. Ranide family of peptides

A large number of antimicrobial peptides isolated from the *Rana* genus are characterized by a large linear segment followed by a C-terminal cationic

loop linked by a disulfide bridge containing 7 amino acids. Examples of these peptides include brevenins [161,162], gaegurins [163], esculentin and ranalexin [164]. The primary structure of brevenin IE is FLPLLAGLAANFLPKIFCKITRKC. The other peptides which have a C-terminal loop have amino acid compositions similar to brevenin IE but vary in length. The C-terminal loop resembles the lipid A binding heptapeptide ring domain of polymyxin B and hence has been presumed to play a role in its antimicrobial activity [162]. An analog of ranalexin in which the C-terminal cysteine was deleted exhibited a 32-fold decrease in antimicrobial activity, in agreement with the above assumption [164]. However, reduction of disulfide bridges in ranalexin and replacement of cysteines did not markedly decrease the activity, suggesting that the disulfide bridge in ranalexin did not play a major role in its activity [165]. The decrease in activity due to deletion of the C-terminal cysteine appears to reflect a crucial role for this residue for its activity rather than a requirement for a disulfide bond. Similar results have been obtained in the case of brevenin IE and gaegurin, which have two cysteine residues linked by a disulfide bridge [166–168].

#### 6.1.2. Bactenecin

Bactenecin, a dodecapeptide from bovine neutrophils, having the sequence RLCRIVVIRVCR exhibits its potent antibacterial activity [169]. The CD spectrum of the peptide in phosphate buffer is typical of peptides with type I  $\beta$ -turn, with a characteristic negative ellipticity at 205 nm [92]. The same structure was also observed in TFE, SDS and liposomes composed of anionic lipids. Computer modeling studies, correlated with the structure indicated by the CD spectra, suggest that the peptide adopts a rigid  $\beta$ -turn loop structure irrespective of its environment. Structure–activity studies on bactenecin have revealed some interesting aspects of its activity. The naturally occurring cyclic peptide possessed activity primarily against Gram-negative bacteria with activity against a few strains of Gram-positive bacteria [92]. The linearized peptide obtained by reduction of the disulfide bridge or replacement of C with S exhibited activity against Gram-positive bacteria with virtually no activity against Gram-negative bacteria except for strains with altered OM. The cyclic

and linear analogs were capable of permeabilizing the outer membrane of Gram-negative bacteria, with the cyclic form more effective than the linearized peptide. However, only the linear analog effectively permeabilized the cytoplasmic membrane. The linear analogs assumed predominantly  $\alpha$ -helical structure in TFE but  $\beta$ -sheet structure in the presence of liposomes or detergent. Increasing the number of positive charges at the N- and C-termini and adding an extra W residue increased the activity of batenecin analogs against Gram-positive and -negative bacteria and also broadened the antimicrobial spectrum [170].

## 6.2. Peptides with two disulfide bridges

Antimicrobial peptides containing four cysteine residues linked by two disulfide bonds with bicyclic structures have been characterized from the hemolymph of the horseshoe crabs *Tachypleus tridentatus* and *Limulus polyphemus* [171–173] as well as from porcine leukocytes [174–176]. These are known as tachyplesins (KWCFRVCYRGICYRRCR-Am, tachyplesin-1), polyphemusins (RRWCFRVCYRGFCYRKCR-Am, polyphemusin-1), and protegrins (RGRLCYCRRRFCVCVGR-Am, PG-1), respectively. These peptides are between 16 and 18 residues in length and adopt an antiparallel  $\beta$ -sheet structure which is stabilized by the two disulfide bridges. All these peptides exhibit broad spectrum antimicrobial activity and appear to function by membrane disruption. They also possess antiviral activity against HIV-1. Studies aimed at understanding the importance of the disulfide bridges for their activity have been carried out on this group of peptides. Studies with tachyplesin analogs, where the SH groups were chemically protected to prevent cyclization or cysteines were replaced by A residues, suggested that the cyclic structure was essential for antimicrobial activity while it might not be crucial for membrane permeabilization [177–180]. Detailed studies have indicated that replacement of the cysteine residues by certain amino acid like A, D and I lead to inactivation, whereas analogs with aromatic residues F and Y and hydrophobic amino acid like L, M and V retained broad spectrum antimicrobial activity [181]. These results suggest subtle influences of the

amino acid side chains on the overall conformation of the analog [179].

Tamamura et al. have attempted to develop less toxic analogs of polyphemusin with improved activity against HIV [181,182]. An analog of polyphemusin II having the replacements [(Y5,12, K7)] was found to be considerably less cytotoxic but retained the antiviral potency of its parent peptide. An analog with only one S–S bridge, RRWCYRKDKPYRK-CR-Am, had antiviral activities comparable to the parent peptide polyphemusin II but was less cytotoxic.

Protegrin adopts  $\beta$ -sheet a structure in aqueous and membrane-mimetic environments whereas the linear variants were unordered in water and helical in membrane-mimetic environments [183]. Hence, the intramolecular disulfide bonds appear to be essential for  $\beta$ -sheet formation and potent antibacterial activity in media comparable to those found in serum and extracellular fluids. Protegrin and its linear variants were active in the absence of high salt at comparable concentrations, but the S–S bridges were necessary for activity at high salt concentrations and for pore formation [184].

Structure–function studies on mammalian defensins involving truncated analogs have not been extensive. The  $\beta$ -hairpin region of rabbit defensin NP-2 (composed of 18 amino acids) has been shown to have antibacterial activity [185].

## 7. Mechanisms of model membrane permeabilization

It is clearly evident that positive charges play an important role in determining antibacterial activity whereas an amphiphilic helical structure is necessary for hemolytic activity. Considerable information about the orientation of peptides on the membrane surface and the possible structure of the pores has emerged from detailed studies on magainin and to a lesser extent on cecropins and defensins. Some recent findings are reviewed in this section. The ability of magainins to permeabilize lipid vesicles was first studied by Matsuzaki et al. by monitoring the release of entrapped calcein from small unilamellar vesicles [186]. It was observed that PS but not PC vesicles were permeabilized by the peptide. By analyzing both

the peptide and lipid concentration dependence of the leakage rate, the affinity of peptide for lipid vesicles and the amount of membrane-bound peptide were evaluated. A binding constant of  $10^5 \text{M}^{-1}$  was observed for PS vesicles. The data did not suggest extensive aggregation of peptide in membranes although 100–300 molecules were apparently bound to one vesicle. While the peptide was unordered in aqueous medium and PC vesicles, a helical conformation was observed in the presence of PS vesicles. The lipid/peptide ratio at which substantial calcein was released was  $\sim 10:1$ , indicating that the peptide did not permeabilize even PS vesicles effectively, unlike the cytolytic peptides, melittin and pardaxin. Magainin–lipid interactions have been extensively investigated [187–201]. Judiciously designed analogs and studies involving CD spectroscopy and calcein release have clarified magainin–lipid interactions to a considerable extent. Studies using systematic double D-amino acid replacement have revealed that upon binding to liposomes, a stable  $\alpha$ -helix appears to be formed between residues 9 and 21 with the N-terminal 1–8 segment forming a flexible  $\alpha$ -helix. Binding to negatively charged vesicles was independent of the content of negative charges and a well-stabilized helix between residues 9–21 was important for this interaction [190]. The charges in the polar face in magainin appear to be optimized for interaction with negatively charged vesicles, as analogs where the angle subtended by the positively charged helix face, as well as those with greater hydrophobic moment than in magainin, were able to permeabilize PC vesicles but could permeabilize PG vesicles less effectively [194,195,197]. The question of magainin aggregation on the membrane surface in the bilayer and the structure of the pore has been the subject of extensive studies. One and two dimensional solid state  $^{15}\text{N}$ -NMR data of specifically labeled  $^{15}\text{N}$  magainin 2 in oriented bilayer samples have been interpreted as the peptide oriented parallel to the membrane surface. An orientation parallel to the bilayer surface at low peptide lipid molar ratios and perpendicular orientation at a high lipid/peptide ratio has been suggested based on oriented CD data [200]. Fluorescence energy transfer experiments involving W16 magainin and  $\text{N}^\alpha$  dansyl fluorophore [196] appear to suggest an orientation parallel to the membrane surface and absence of association of magainin mol-

ecules on the membrane surface. However, a model for the magainin pore proposed by Matsuzaki and co-workers involves oligomerization of peptides with 4–5 molecules involved in forming a pore [188,189]. The aggregation number of 5 has been arrived at by analysis of the calcein release profiles as a function of peptide concentration and accounting for translocation of peptide molecules to the inner leaflet. Studies on planar bilayers have indicated an aggregation number in the range of 3–6 [74]. The variation in the number of molecules in the aggregates suggests that well-defined aggregates are perhaps not formed and the experimentally determined values are averages. The model proposed for membrane permeabilization by magainin involves binding of magainin molecules on the membrane surface followed by formation of a pore composed of peptide–lipid complexes, which on disintegration leads to translocation of magainin molecules to the inner leaflet [189,191–193]. The model involving lipid head groups as part of the channel has also been proposed by Ludtke et al. [201]. It is evident that this model, referred to as toroidal model, proposed for the magainin pore is considerably different from those of gramicidin A and alamethicin. The rapid flip-flop of phospholipids induced by magainin [191] is presumed to arise due to the formation of toroidal pores.

The question of the specificity of magainin for prokaryotic membranes has been addressed. The inability of magainin to bind to zwitterionic vesicles has been suggested as a reason for its lack of hemolytic activity as erythrocytes do not have negatively charged lipids on the outer monolayer. Magainin can cause lysis when a negative inside potential is generated. Hence, lysis of erythrocytes does not occur due to lack of membrane potential [202]. Another viewpoint that has been presented is that peptide–cholesterol interaction in the membrane inhibit the formation of structures capable of lysis. The specific activity against tumorigenic cells could arise as there is 3–8-fold more of the negatively charged lipid, PS, on the cell surface of transformed cells as compared to normal cells, and magainins could bind to these molecules and cause the permeabilization of the plasma membrane [203].

Magainin 2 and a related frog skin peptide PGLa (see Table 1 for sequence) have been shown to exhibit synergism in their activities [204,205]. The syn-

ergism is presumed to arise as a result of the formation of heterosupra molecular complexes which form pores at a faster rate as compared to magainins and which are also moderately more stable than the magainin pores.

The binding of the 34-residue frog skin peptide dermaseptin with model membranes has been investigated by monitoring the fluorescence of the extrinsic fluorophores 7-nitrobenz-2-oxa-1,3-diazole-4-yl (NBD), fluorescein or rhodamine-labeled peptides in the presence of lipid vesicles of varying composition [206]. The surface partition coefficients derived from the binding isotherms indicated that the peptide associated with negatively charged vesicles like PG to a fourfold greater extent than zwitterionic vesicles. The peptide also permeabilized PG containing vesicles more effectively than PC vesicles. It has been proposed that the dermaseptin molecules bind to the anionic membrane surface from an initial random conformation and binding induces a helical conformation which is amphiphilic. This conformation has been proposed to generate a dipole moment along the helix axis equivalent to a half-negative charge on the C-terminus and a half-positive charge on the N-terminus. The opposed electric fields between this dipole and the difference of potential across the plasma membrane results in the insertion of the peptide chain into the lipid bilayer in a transverse orientation which would perturb the bilayer structure. Aggregation of several monomers would result in additional perturbation resulting in permeabilization by pore formation. The N-terminal segment is presumed to be associated with the bilayer [130].

The interaction of mammalian cecropin P1 to model membranes has been studied by ATR-FTIR spectroscopy and molecular dynamics simulations [72] and fluorescence spectroscopy [207]. The data have been interpreted in terms of a parallel orientation at the membrane surface. Since the acyl chains were not perturbed, it appears that the peptide does not penetrate the hydrophobic core. The peptide was found to associate with zwitterionic as well as negatively charged vesicles. The model for cecropin P1-induced membrane permeabilization involves destabilization of the phospholipid packing leading to disintegration as a result of the formation of a layer of peptide monomers on the membrane surface.

Cecropin A has been shown to dissipate membrane potential at low concentrations and cause the release of calcein from lipid vesicles composed of both zwitterionic and cationic lipids. At low concentrations when depolarization of vesicle membrane occurs, no release of calcein was observed. Although cecropin A binds more strongly to anionic lipids, this does not result in greater membrane permeabilization of lipid vesicles as compared to membranes with zwitterionic lipids. The peptide could permeabilize vesicles containing cholesterol [208]. Cecropin B2, a variant of cecropin A, also bound to negatively charged vesicles more effectively, but unlike cecropin A, permeabilized them to a greater extent than zwitterionic vesicles [35]. Cecropin B2, like cecropin A, is also presumed to bind to phospholipids as monomers and orient parallel to the membrane surface. The binding of cecropin B to lipid vesicles was recently investigated by surface plasmon resonance and stop flow measurement of CD and fluorescence [36]. These studies indicated that stronger binding did not result in increased membrane permeabilization. Helix formation appeared to be a one-step process whereas vesicle lysis was a two-step process. A peptide composed of the N-terminal regions 1–8 of cecropin A and 1–18 of melittin showed preferential activity towards PS vesicles. A high peptide stoichiometry was required for vesicle disruption as for magainin and cecropins [209].

Defensins, which have three S–S bridges, have also been shown to form weakly anion-selective voltage-dependent channels independent of lipid composition [77]. Measurement of the dependence of the logarithm of membrane conductance on the logarithm of defensin NP-1 concentration indicated an aggregation number of 2–4. The defensins carry a net positive charge between +2 to +9, suggesting that their membrane activity would be highly variable. An investigation on human defensins HNP-1 and HNP-2 (net charge = +3) indicated binding to PG vesicles but not to PC vesicles [210,211]. The PG vesicles aggregated in the presence of HNP-2. However, no release of vesicular contents was detected although lipid mixing was detected. This was attributed to hemifusion. Based on the analysis of the release of trapped markers, it has been proposed that HNP-2 forms multimeric pores in lipid bilayers composed of 6–8 bilayer-spanning defensin dimers in the form of

an annulus around an aqueous channel of  $\sim 25$  Å diameter. HNP-1 could also induce fusion of PG-containing vesicles [210]. Rabbit defensins also have the ability to bind to lipid vesicles composed of POPG or POPG with neutral phospholipids and but could cause fusion of only POPG phospholipid vesicles [212]. While rabbit defensin NP-4 did not cause leakage of vesicle contents, the other defensins (NP-1, NP-2, NP-3A, NP-3B and NP-5) caused release of vesicle contents in a graded manner, as against the ‘all or none’ mechanism of HNP-2. In another study, Histova et al. have shown that permeabilization of large unilamellar vesicles made from *E. coli* lipid extracts by rabbit defensins was considerably different from that of composed of pure phospholipids [213]. Cardiolipin appeared to play a key role as no leakage was observed when only PG and PE were present. Maximal release was observed at a lipid/peptide molar ratio of  $\sim 40:1$  with the *E. coli* lipids. The release with synthetic lipids occurred only at a much lower lipid/peptide ratio. Based on their data, the authors have concluded that rabbit defensins do not form pores like human defensin. Differential scanning microcalorimetry studies have indicated that the defensin HNP-2 affected the phase behavior of molecular membranes composed of lipids mimicking bacterial lipids but not erythrocyte lipids [214]. Insect defensin A, which has helical and  $\beta$ -sheet segments, was also shown to interact preferentially with anionic lipids [215].

Tachyplesin 1, like defensin, also showed specific interaction with PG vesicles causing the release of entrapped calcein [178]. Maximal release was observed at lipid/peptide ratio of  $\sim 50:1$ . The molecule was located below the head-group region. Although tachyplesin 1 is not amphiphilic in aqueous environment, it has been proposed that interaction with lipid vesicles induces amphiphilic structure. Tachyplesin 1 without the S–S bridge also associated with lipid vesicles causing fusion/aggregation. However, membrane permeabilization was attributed to disruption of lipid organization rather than pore formation.

## 8. Conclusions and perspectives

Extensive studies on endogenous host-defense peptides, as well as on peptides derived from them, in-

dicate that the presence of positive charges is perhaps the most important determinant for activity. However, the number of positive charges required for activity is variable. Although linear antibacterial peptides do tend to form amphiphilic helical structures in hydrophobic environments, antibacterial activity has been observed even when only a small fraction of molecules populate the helical conformation. In fact, when peptides possessing both antibacterial and hemolytic activities were engineered so as to decrease helical propensity, the antibacterial activity or spectrum were largely unaffected whereas there was considerable loss in hemolytic activity. The common structural feature in antibacterial peptides containing two and three disulfide bridges is a  $\beta$ -sheet structure. However, in the two disulfide bridge-containing peptides, antibacterial activity is observed in the linearized peptides, though to a lower extent as compared to the parent peptide. Hence, it is evident that requirements of charge and amphiphilic helical structure or  $\beta$ -structure for antimicrobial activity are not stringent.

Killing of bacteria by membrane permeabilization appears to be the mechanism of action of the antibacterial peptides described in this review. The killing of bacteria appears to be rapid and multiple mechanisms of action, especially involving perturbation of cellular metabolism, are yet to be established unequivocally. Although antimicrobial peptides do permeabilize lipid vesicles, the lipid/peptide ratios for effective permeabilization are low, suggesting that membrane permeabilization probably does not occur as a result of pores formed by aggregates of peptides. A model for the manner in which the bacterial membranes are permeabilized is shown in Fig. 5. The membrane surface of bacteria composed of LPS or peptidoglycans are destabilized, resulting in generation of a pathway for peptides to enter the cells and bind to the bacterial plasma membrane. Binding of the cationic peptides to the negatively charged membrane surface results in membrane destabilization which could cause defects through which the bacterial membrane potential could be dissipated. It is also likely that some peptides like the magainins form toroidal pores composed of lipid molecules associated with peptides.

The bacterial plasma membrane contains enzymes necessary for respiration. Even slight damage to the

membrane can result in loss of respiratory function. However, lysis of erythrocytes involves creation of pores through which ions are able to leak. When the capacity of the  $\text{Na}^+$  pump to prevent excessive entry of  $\text{Na}^+$ ,  $\text{Cl}^-$  and water is exceeded, colloid osmotic lysis occurs [216]. Hence, peptides that can effectively form pores exhibit both antibacterial and hemolytic activities. Peptides that do not form pores effectively, as a result of which the  $\text{Na}^+$  pump can overcome osmotic imbalance, exhibit selective antibacterial activity as the bacterial membranes would be much more susceptible to membrane damage.

It is thus evident that the mechanism of action of antibacterial peptides that act by permeabilizing membranes can to a large extent be rationalized on the basis of biophysical principles.

There have been recent reports of resistance to cationic antibacterial peptides in *Salmonella*, *E. coli* and *Y. enterocolitica*, and *S. aureus* [99,100,217–219]. Resistance in *Salmonella* appears to stem from increased acylation of lipid A, the major component of the outer leaflet of the outer membrane [99]. The resistance of *B. bronchiseptica* to cationic antimicrobial peptides is presumed to arise due to the structure of lipopolysaccharide, which prevents effective membrane permeabilization [100]. In *S. aureus*, lower sensitivity to cationic antibacterial peptides appears to arise as a result of altered structure of teichoic acids, a major component of the Gram-positive cell wall [219]. Hence, the design of effective membrane-active antimicrobial peptides will be a continuous challenge.

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## References

- [1] J. Kyte, R.F. Doolittle, *J. Mol. Biol.* 157 (1982) 105–132.
- [2] D.M. Engelman, T.A. Steitz, A. Goldman, *Annu. Rev. Biophys. Biophys. Chem.* 15 (1986) 321–353.
- [3] G. von Heijne, *Annu. Rev. Biophys. Biomol. Struct.* 23 (1994) 167–192.
- [4] R.A.F. Reithmeier, *Curr. Opin. Struct. Biol.* 5 (1995) 491–500.
- [5] W.C. Wimley, S.H. White, *Nat. Struct. Biol.* 3 (1996) 842–848.
- [6] C.M. Deber, S.-C. Li, *Biopolymers (Pept. Sci.)* 37 (1995) 295–318.
- [7] S.H. White, W.C. Wimley, *Biochim. Biophys. Acta* 1376 (1998) 339–352.
- [8] P.C. Biggin, M.S.P. Sansom, *Biophys. Chem.* 76 (1999) 161–183.
- [9] J.A. Killian, *Biochim. Biophys. Acta* 1113 (1992) 391–425.
- [10] O.S. Andersen, C. Nielsen, A.M. Maer, J.A. Lundback, M. Goulian, R.E. Koeppe II, *Methods Enzymol.* 294 (1999) 208–224.
- [11] R. Nagaraj, P. Balaram, *Acc. Chem. Res.* 14 (1981) 356–362.
- [12] D.S. Cafiso, *Annu. Rev. Biophys. Biomol. Struct.* 23 (1994) 141–165.
- [13] M.S.P. Sansom, *Prog. Biophys. Mol. Biol.* 55 (1991) 139–235.
- [14] H.G. Boman, *Cell* 65 (1991) 205–207.
- [15] R.I. Lehrer, A.K. Lichtenstein, T. Ganz, *Annu. Rev. Immunol.* 11 (1993) 105–128.
- [16] C.L. Bevins, M. Zasloff, *Annu. Rev. Biochem.* 59 (1990) 395–414.
- [17] P. Nicolas, A. Mor, *Annu. Rev. Microbiol.* 49 (1995) 227–304.
- [18] H.G. Boman, *Annu. Rev. Immunol.* 13 (1995) 61–92.
- [19] D. Barra, M. Simmaco, *TIBTECH* 13 (1995) 205–209.
- [20] M. Simmaco, G. Mignogna, D. Barra, *Biopolymers (Pept. Sci.)* 47 (1998) 435–450.
- [21] J.A. Hoffman, C. Hetru, *Immunol. Today* 13 (1992) 411–415.
- [22] W.F. Broekaert, F.R.G. Terras, B.P.A. Cammue, R.W. Osborn, *Plant Physiol.* 108 (1995) 1353–1358.
- [23] G. Saberwal, R. Nagaraj, *Biochim. Biophys. Acta* 1197 (1994) 109–131.
- [24] W.L. Maloy, U.P. Kari, *Biopolymers (Pept. Sci.)* 37 (1995) 105–122.
- [25] S.H. White, W.C. Wimley, M.E. Selsted, *Curr. Opin. Struct. Biol.* 5 (1995) 521–527.
- [26] B. Bechinger, *J. Membr. Biol.* 156 (1997) 191–211.
- [27] K. Matsuzaki, *Biochim. Biophys. Acta* 1376 (1998) 391–400.
- [28] R. Nagaraj, *Curr. Sci.* 72 (1997) 819–825.
- [29] C.E. Dempsey, *Biochim. Biophys. Acta* 1031 (1990) 143–161.
- [30] Y. Shai, *Toxicology* 87 (1994) 109–129.
- [31] D. Andreu, R.B. Merrifield, H. Steiner, H.G. Boman, *Biochemistry* 24 (1985) 1683–1688.
- [32] H. Steiner, D. Andreu, R.B. Merrifield, *Biochim. Biophys. Acta* 939 (1988) 260–266.
- [33] J. Fink, R.B. Merrifield, A. Boman, H.G. Boman, *J. Biol. Chem.* 264 (1989) 6260–6267.
- [34] J. Fink, A. Boman, H.G. Boman, R.B. Merrifield, *Int. J. Pept. Protein Res.* 33 (1989) 412–421.
- [35] E. Gazit, W.-J. Lee, P.T. Brey, Y. Shai, *Biochemistry* 33 (1994) 10681–10692.

- [36] W. Wang, D.K. Smith, K. Moulding, H.M. Chen, *J. Biol. Chem.* 273 (1998) 27438–27448.
- [37] R.B. Merrifield, L.D. Vizioli, H.G. Boman, *Biochemistry* 21 (1982) 5020–5031.
- [38] D. Wade, D. Andreu, S.A. Mitchell, A.M.V. Silveira, A. Boman, H.G. Boman, R.B. Merrifield, *Int. J. Pept. Protein Res.* 40 (1992) 429–436.
- [39] Z. Oren, Y. Shai, *Biopolymers (Pept. Sci.)* 47 (1998) 451–463.
- [40] R.W. Woody, *Peptides* 7 (1985) 15–114.
- [41] W.C. Johnson Jr., *Annu. Rev. Biophys. Chem.* 17 (1988) 145–166.
- [42] W.C. Johnson, *Proteins* 7 (1990) 205–214.
- [43] A. Perczel, M. Hollosi, G. Tushady, G.D. Fasman, *Protein Eng.* 4 (1991) 669–679.
- [44] A. Perczel, K. Park, G.D. Fasman, *Anal. Biochem.* 203 (1992) 83–93.
- [45] M. Sreerama, R.W. Woody, *J. Mol. Biol.* 242 (1994) 497–507.
- [46] N.J. Greenfield, *Anal. Biochem.* 235 (1996) 1–10.
- [47] K. Wuthrich, *Methods Enzymol.* 177 (1989) 125–131.
- [48] T.A. Holak, A. Engstrom, P.J. Kraulis, G. Lindberg, H. Bennis, T. Alwyn-Jones, A.M. Gronenborn, G.M. Clore, *Biochemistry* 27 (1988) 7620–7629.
- [49] D. Sipos, M. Andersson, A. Ehrenberg, *Eur. J. Biochem.* 209 (1992) 163–169.
- [50] D. Marion, M. Zasloff, A. Bax, *FEBS Lett.* 227 (1988) 21–26.
- [51] B. Bechinger, M. Zasloff, S. Opella, *J. Protein Sci.* 2 (1993) 2077–2084.
- [52] D.J. Hirsh, J. Hammer, W.L. Maloy, J. Blazyk, J. Schaefer, *Biochemistry* 35 (1996) 12733–12741.
- [53] H.J. Dyson, G. Merutka, J.P. Waltho, R.A. Lehrer, P.E. Ubricht, *J. Mol. Biol.* 226 (1992) 795–817.
- [54] J. Kemmink, T.E. Creighton, *Biochemistry* 34 (1995) 12630–12635.
- [55] J.J. Yang, M. Buck, M. Pitkeathly, M. Kotik, D.T. Haynie, C.M. Dobson, S.E. Eadford, *J. Mol. Biol.* 252 (1995) 483–491.
- [56] K.A. Bobin, M. Pitkeathly, A. Mirankes, L.J. Smith, C.M. Dobson, *J. Mol. Biol.* 261 (1996) 443–453.
- [57] M.T. Reymond, S. Hwo, B. Duggan, P.E. Ubricht, J.H. Dyson, *Biochemistry* 36 (1997) 5234–5244.
- [58] A.C. Bach, M.E. Selsted, A. Pardi, *Biochemistry* 26 (1987) 4389–4397.
- [59] J.J. Skalicky, M.E. Selsted, A. Pardi, *Proteins* 20 (1994) 52–67.
- [60] A. Pardi, D.R. Hare, M.E. Selsted, R.D. Morrison, D.A. Bassolino, A.C. Bach, *J. Mol. Biol.* 201 (1988) 625–636.
- [61] X.-L. Zhang, M.E. Selsted, A. Pardi, *Biochemistry* 31 (1992) 11348–11356.
- [62] A. Pardi, X.-L. Zhang, M.E. Selsted, J.J. Skalicky, P.F. Yip, *Biochemistry* 31 (1992) 11357–11364.
- [63] G.R. Zimmermann, P. Legault, M.E. Selsted, A. Pardi, *Biochemistry* 34 (1995) 13663–13671.
- [64] H. Hanzawa, I. Shimada, T. Kuzuhara, H. Komand, D. Kohda, F. Inagaki, S. Natori, Y. Hrata, *FEBS Lett.* 269 (1990) 413–420.
- [65] J.M. Bonmatin, J.L. Bomat, Z. Gallet, F. Vovelle, M. Ptak, J.M. Reichhart, J.A. Hoffmann, E. Keppi, M. Legrain, T. Achstetter, *J. Biomol. NMR* 2 (1992) 235–256.
- [66] M. Bruix, M.A. Jimenez, J. Santoro, C. Gonzalez, F.J. Wilila, E. Mendez, M. Rico, *Biochemistry* 132 (1993) 715–724.
- [67] K. Kawano, T. Yoneya, T. Miyala, K. Yoshikawa, F. Tokunaga, Y. Terada, S. Ewanaga, *J. Biol. Chem.* 265 (1990) 15365–15367.
- [68] C.P. Hill, J. Yee, M.E. Selsted, D. Eisenberg, *Science* 251 (1991) 1481–1485.
- [69] R.W. Williams, R. Starman, K.M.P. Taylor, K. Bable, T. Beeler, M. Zasloff, D. Covell, *Biochemistry* 29 (1990) 4490–4496.
- [70] M. Jackson, M.M. Mantsch, J.H. Spencer, *Biochemistry* 31 (1992) 7289–7293.
- [71] W.T. Heller, A.J. Waring, R.T. Lehrer, H.W. Huang, *Biochemistry* 37 (1998) 17331–17338.
- [72] E. Gazit, I.R. Miller, P.C. Biggin, M.S.P. Sansom, Y. Shai, *J. Mol. Biol.* 258 (1996) 860–870.
- [73] M. Zasloff, *Proc. Natl. Acad. Sci. USA* 84 (1987) 5449–5453.
- [74] H. Duclouhier, G. Molle, G. Spach, *Biophys. J.* 56 (1989) 1017–1021.
- [75] R.A. Cruciani, J.L. Barker, M. Zasloff, H.-C. Chen, O. Colamonici, *Proc. Natl. Acad. Sci. USA* 88 (1991) 3792–3796.
- [76] B. Christensen, J. Fink, R.B. Merrifield, D. Mauzerall, *Proc. Natl. Acad. Sci. USA* 85 (1988) 5072–5076.
- [77] B.L. Kagan, M.E. Selsted, T. Ganz, R.I. Lehrer, *Proc. Natl. Acad. Sci. USA* 87 (1990) 210–214.
- [78] S. Cociancich, A. Ghazi, C. Hetru, J.A. Hoffmann, L. Letellier, *J. Biol. Chem.* 268 (1993) 19239–19245.
- [79] B. Lugtenberg, O. Van Alphen, *Biochim. Biophys. Acta* 737 (1983) 51–115.
- [80] M. Vaara, *Microbiol. Rev.* 56 (1992) 395–411.
- [81] F.R. Rana, J. Blazyk, *FEBS Lett.* 293 (1991) 11–15.
- [82] F.R. Rana, E.A. Macias, C.M. Sultany, M.C. Modzrakowski, J. Blazyk, *Biochemistry* 30 (1991) 5858–5866.
- [83] K. Matsuzaki, K. Sugishita, K. Miyajima, *FEBS Lett.* 449 (1999) 221–224.
- [84] N. Sitaram, V. Krishnakumari, R. Nagaraj, *FEBS Lett.* 303 (1992) 265–268.
- [85] M. Vaara, W.Z. Plachy, H. Nikaido, *Biochim. Biophys. Acta* 1024 (1990) 152–156.
- [86] R.I. Lehrer, A. Barton, T. Ganz, *J. Immunol. Methods* 108 (1988) 153–158.
- [87] J.G. Sawyer, N.L. Martin, R.E.W. Hancock, *Infect. Immun.* 56 (1988) 693–698.
- [88] R.I. Lehrer, A. Barton, K.A. Daher, S.S.L. Harwig, T. Ganz, M.E. Selsted, *J. Clin. Invest.* 84 (1989) 553–561.
- [89] B. Skerlavaj, D. Romeo, R. Gennaro, *Infect. Immun.* 58 (1990) 3724–3730.
- [90] T.J. Falla, N. Karunaratne, R.E.W. Hancock, *J. Biol. Chem.* 271 (1996) 19298–19303.
- [91] T.J. Falla, R.E.W. Hancock, *Antimicrob. Agents Chemother.* 41 (1997) 771–775.

- [92] M. Wu, R.E.W. Hancock, *J. Biol. Chem.* 274 (1999) 29–35.
- [93] J.-T. Oh, Y. Cajal, P.S. Dhurjati, T.K. Van Dyk, M.K. Jain, *Biochim. Biophys. Acta* 1415 (1998) 235–245.
- [94] M. Shimoda, K. Ohki, Y. Shimamoto, O. Kohashi, *Infect. Immun.* 63 (1995) 2886–2891.
- [95] Z. Oren, Y. Shai, *Eur. J. Biochem.* 237 (1996) 303–310.
- [96] Y. Shai, Z. Oren, *J. Biol. Chem.* 271 (1996) 7305–7308.
- [97] S.C. Chan, W.L. Yau, W. Wang, D.K. Smith, F.-S. Sheu, H.M. Chen, *J. Pept. Sci.* 4 (1998) 413–425.
- [98] M.R. Yeaman, A.S. Bayer, S.-P. Koo, W. Foss, P.M. Sullam, *J. Clin. Invest.* 101 (1998) 178–187.
- [99] L. Guo, K.B. Lim, C.M. Poduje, M. Daniel, J.S. Gunn, M. Hackett, S.I. Miller, *Cell* 95 (1998) 189–198.
- [100] A. Banemann, H. Deppisch, R. Gross, *Infect. Immun.* 66 (1998) 5607–5612.
- [101] Y.-Q. Xiong, M.R. Yeaman, A.S. Bayer, *Antimicrob. Agents Chemother.* 43 (1999) 1111–1117.
- [102] D. Wade, A. Boman, B. Wahlin, C.M. Drain, D. Andreu, H.G. Boman, R.B. Merrifield, *Proc. Natl. Acad. Sci. USA* 87 (1990) 4761–4765.
- [103] R. Bessalle, A. Kapitkovsky, A. Gorea, I. Shalit, M. Fridkin, *FEBS Lett.* 274 (1990) 151–155.
- [104] M.M. Welling, P.S. Hiemstra, M.T. vanden Barselaar, A. Paulusma-Annema, P.H. Nibbering, E.K.J. Pauwels, W. Calame, *J. Clin. Invest.* 102 (1998) 1583–1590.
- [105] J.W. Lillard Jr., P.N. Boyaka, O. Chertov, J.J. Oppenheim, J.R. McGhee, *Proc. Natl. Acad. Sci. USA* 96 (1999) 651–656.
- [106] E.S.P. Reddy, P.M. Bhargava, *Nature* 279 (1979) 725–728.
- [107] N. Sitaram, V. Krishnakumari, P.M. Bhargava, *FEBS Lett.* 201 (1986) 233–236.
- [108] N. Sitaram, R. Nagaraj, *BioEssays* 17 (1995) 415–422.
- [109] K.H. Scheit, E.S.P. Reddy, P.M. Bhargava, *Nature* 279 (1979) 728–731.
- [110] S. Shivaji, K.H. Scheit, P.M. Bhargava, *Proteins of Seminal Plasma*, Wiley, New York, 1989, pp. 337–349.
- [111] T.R. Murti, S.A. Chandani, A. Khar, G.L. Reddy, P.M. Bhargava, *Biochim. Biophys. Acta* 1221 (1994) 109–114.
- [112] N. Sitaram, Ph.D Thesis, Jawaharlal Nehru University, New Delhi, 1993.
- [113] K. Gietzen, H.-J. Galla, *Biochem. J.* 230 (1985) 277–280.
- [114] H.J. Galla, M. Warncke, K.H. Scheit, *Eur. Biophys. J.* 12 (1985) 211–216.
- [115] T. Maurer, H. Rutergans, *Eur. J. Biochem.* 220 (1994) 111–116.
- [116] N. Sitaram, C. Subbalakshmi, R. Nagaraj, *FEBS Lett.* 328 (1993) 239–242.
- [117] N. Sitaram, R. Nagaraj, *J. Biol. Chem.* 265 (1990) 10438–10442.
- [118] N. Sitaram, C. Subbalakshmi, V. Krishnakumari, R. Nagaraj, *FEBS Lett.* 400 (1997) 289–292.
- [119] N. Sitaram, R. Nagaraj, *Biochemistry* 32 (1993) 3124–3130.
- [120] N. Sitaram, C. Subbalakshmi, R. Nagaraj, *Int. J. Pept. Protein Res.* 46 (1995) 166–173.
- [121] N. Sitaram, M. Chandy, V.N.R. Pillai, R. Nagaraj, *Antimicrob. Agents Chemother.* 36 (1992) 2468–2472.
- [122] E. Bikshapathy, N. Sitaram, R. Nagaraj, *J. Pept. Res.* 53 (1999) 47–55.
- [123] K. Krishnakumari, A. Sharada Devi, N. Sitaram, R. Nagaraj, *J. Pept. Res.* (1999) in press.
- [124] E. Bikshapathy, N. Sitaram, R. Nagaraj, *Protein Pept. Lett.* 6 (1999) 67–71.
- [125] S. Thennarasu, R. Nagaraj, *Biopolymers* 41 (1999) 635–645.
- [126] S. Thennarasu, R. Nagaraj, *Protein Eng.* 9 (1996) 1219–1224.
- [127] S. Thennarasu, R. Nagaraj, *Int. J. Pept. Protein Res.* 46 (1995) 480–486.
- [128] A. Mor, V.H. Nguyen, A. Delfour, D. Miglore, P. Nicolas, *Biochemistry* 30 (1991) 8824–8830.
- [129] A. Mor, K. Hani, P. Nicolas, *J. Biol. Chem.* 269 (1994) 31635–31641.
- [130] A. Mor, P. Nicolas, *J. Biol. Chem.* 269 (1994) 1934–1939.
- [131] P.J. Coote, C.D. Holyoak, D. Bracey, D.P. Ferdinando, J.A. Pearce, *Antimicrob. Agents Chemother.* 42 (1998) 2160–2170.
- [132] J.F. Fennel, W.H. Shipman, L. Cole, *J. Proc. Soc. Exp. Biol. Med.* 127 (1968) 707–710.
- [133] T.C. Terwilliger, L. Weissman, D. Eisenberg, *Biophys. J.* 37 (1982) 353–361.
- [134] E. Schroder, K. Lubke, M. Lehmann, I. Beetz, *Experientia* 27 (1971) 764–765.
- [135] C.R. Dawson, A.F. Drake, J. Helliwell, R.C. Hider, *Biochim. Biophys. Acta* 510 (1978) 75–86.
- [136] S.E. Blondelle, R.A. Houghten, *Biochemistry* 30 (1991) 4671–4678.
- [137] E. Perez-Paya, R.A. Houghten, S.E. Blondelle, *J. Biol. Chem.* 270 (1995) 1048–1056.
- [138] P. Juvvadi, S. Vumman, R.B. Merrifield, *J. Am. Chem. Soc.* 118 (1996) 8989–8997.
- [139] Z. Oren, Y. Shai, *Biochemistry* 36 (1997) 1826–1835.
- [140] E. Habermann, H. Kowallek, *Physiol. Chem.* 351 (1970) 884–890.
- [141] S.E. Blondelle, R.A. Houghten, *Pept. Res.* 4 (1991) 12–18.
- [142] A.K. Ghosh, R. Rukmini, A. Chattopadhyay, *Biochemistry* 36 (1997) 14291–14305.
- [143] A.J. Weaver, M.D. Kemple, F.G. Prendergast, *Biochemistry* 28 (1989) 8614–8623.
- [144] V.S. Gevod, K.S. Birdi, *Biophys. J.* 45 (1984) 1079–1083.
- [145] C. Subbalakshmi, R. Nagaraj, N. Sitaram, *FEBS Lett.* 448 (1999) 62–66.
- [146] H.G. Boman, D. Wade, I.A. Boman, B. Wahlin, R.B. Merrifield, *FEBS Lett.* 259 (1989) 103–106.
- [147] D. Andreu, J. Ubach, A. Boman, B. Wahlin, D. Wade, R.B. Merrifield, H.G. Boman, *FEBS Lett.* 296 (1992) 190–194.
- [148] M.E. Selsted, M.J. Novotny, W.L. Morris, Y.-Q. Tang, W. Smith, J.S. Cullor, *J. Biol. Chem.* 267 (1992) 4292–4295.
- [149] M. Zanetti, R. Gennaro, D. Romeo, *FEBS Lett.* 374 (1995) 1–5.
- [150] R.J. Van Abel, Y.-Q. Tang, V.S.V. Rao, C.H. Dobbs, D.



- Tran, G. Barany, M.E. Selsted, *Int. J. Pept. Protein Res.* 45 (1995) 401–409.
- [151] A. Giacometti, O. Cirioni, G. Greganti, M. Quarta, G. Scalise, *Antimicrob. Agents Chemother.* 42 (1998) 3320–3324.
- [152] W.E. Robinson Jr., B. McDougall, D. Tran, M.E. Selsted, *J. Leukocyte Biol.* 63 (1998) 94–100.
- [153] I. Ahmad, W.R. Perkins, D.M. Lupan, M.E. Selsted, A.S. Janoff, *Biochim. Biophys. Acta* 1237 (1995) 109–114.
- [154] C. Subbalakshmi, V. Krishnakumari, R. Nagaraj, N. Sitaram, *FEBS Lett.* 395 (1996) 48–52.
- [155] C. Subbalakshmi, N. Sitaram, *FEMS Microbiol. Lett.* 160 (1998) 91–96.
- [156] C. Subbalakshmi, V. Krishnakumari, N. Sitaram, R. Nagaraj, *J. Biosci.* 23 (1998) 9–13.
- [157] A.S. Ladokhin, M.E. Selsted, S.H. White, *Biophys. J.* 72 (1997) 794–805.
- [158] Y.-B. Lim, J.-C. Pyun, J.-S. Park, *J. Biochem. Mol. Biol.* 30 (1997) 229–233.
- [159] C. Lawyer, S. Pai, M. Watabe, P. Borgia, T. Mashimo, L. Eagleton, K. Watabe, *FEBS Lett.* 390 (1996) 95–98.
- [160] C.L. Guerneve, M. Seigneuret, D. Marion, *Arch. Biochem. Biophys.* 360 (1998) 179–186.
- [161] N. Morikawa, K. Hagiwara, T. Nakajima, *Biochem. Biophys. Res. Commun.* 189 (1992) 184–190.
- [162] M. Simmaco, G. Mignogna, D. Barra, F. Bossa, *J. Biol. Chem.* 269 (1994) 11956–11961.
- [163] J.M. Park, M.-E. Jung, B.J. Lee, *Biochem. Biophys. Res. Commun.* 205 (1994) 948–954.
- [164] D.P. Clark, S. Durell, L.W. Maloy, M. Zasloff, *J. Biol. Chem.* 269 (1994) 10849–10855.
- [165] E. Vignal, A. Chavanieu, P. Roch, L. Chiche, G. Grassay, B. Calas, A. Aumelas, *Eur. J. Biochem.* 253 (1998) 221–228.
- [166] M.Y. Kwon, S.-Y. Hong, K.-H. Lee, *Biochim. Biophys. Acta* 1387 (1998) 239–248.
- [167] J.-Y. Suh, K.-H. Lee, S.-W. Chi, S.-Y. Hong, B.-W. Choi, H.-M. Moon, B.-S. Choi, *FEBS Lett.* 392 (1996) 309–312.
- [168] S. Kim, J.-Y. Kim, B.-J. Lee, S.J. Kim, *J. Biochem. Mol. Biol.* 32 (1999) 12–19.
- [169] D. Romeo, B. Skerlavaj, M. Bolognesi, R. Gennaro, *J. Biol. Chem.* 263 (1988) 9573–9575.
- [170] M. Wu, R.E.W. Hancock, *Antimicrob. Agents Chemother.* 43 (1999) 1274–1276.
- [171] T. Nakamura, H. Furunaka, T. Miyata, F. Tokunaga, T. Muta, S. Iwanaga, M. Niwa, T. Takao, Y. Shimonishi, *J. Biol. Chem.* 263 (1988) 16709–16713.
- [172] T. Miyata, F. Tokunaga, T. Yoneya, K. Yoshikawa, S. Iwanaga, M. Niwa, T. Takao, Y. Shimonishi, *J. Biochem.* 106 (1989) 663–668.
- [173] T. Muta, T. Fujimoto, H. Nakajima, S. Iwanaga, *J. Biochem.* 108 (1990) 261–266.
- [174] V.N. Kokryakov, S.S.L. Harwig, E.A. Panyutich, A.A. Shevehenko, G.M. Aleshina, O.V. Shamova, H.A. Korneva, R.I. Lehrer, *FEBS Lett.* 327 (1993) 231–236.
- [175] O.A. Migorodskaya, A.A. Shevehenko, K.O. Abdalla, I.V. Chernushevich, T.A. Egorov, A.X. Musoliamov, V.N. Kokryakov, O.V. Shamova, *FEBS Lett.* 330 (1993) 339–342.
- [176] S. Harwig, K.L.T. Swiderek, T.D. Lee, R.I. Lehrer, *J. Pept. Sci.* 3 (1995) 207–215.
- [177] H. Tamamura, R. Ikoma, M. Niwa, S. Funakoshi, T. Murakami, N. Fujii, *Chem. Pharm. Bull.* 41 (1993) 978–980.
- [178] K. Matsuzaki, S. Yoneyama, N. Fujii, K. Miyajima, K. Yamada, Y. Kirino, K. Anzai, *Biochemistry* 36 (1997) 9799–9806.
- [179] O. Oishi, S. Yamashita, E. Nishimoto, S. Lee, G. Sugihara, M. Ohno, *Biochemistry* 36 (1997) 4352–4359.
- [180] A.G. Rao, *Arch. Biochem. Biophys.* 361 (1999) 127–134.
- [181] H. Tamamura, M. Waki, M. Imai, A. Otaka, T. Ibuka, K. Waki, K. Miyamoto, A. Matsumoto, T. Murakami, H. Nakashima, N. Yamamoto, N. Fujii, *Bioorganic. Med. Chem.* 6 (1998) 473–479.
- [182] H. Tamamura, R. Arakaki, H. Funakoshi, M. Imai, A. Otaka, T. Ibuka, H. Nakashima, T. Murakami, M. Waki, A. Matsumoto, N. Yamamoto, N. Fujii, *Bioorg. Med. Chem.* 6 (1998) 231–238.
- [183] S.S.L. Harwig, A. Waring, H.J. Yang, Y. Cho, L. Tan, R.I. Lehrer, *Eur. J. Biochem.* 240 (1996) 352–357.
- [184] K. Chiche, E. Despauze, G. Grassy, B. Calas, A. Chavanieu, *FEBS Lett.* 383 (1996) 93–98.
- [185] S. Thennarasu, R. Nagaraj, *Biochem. Biophys. Res. Commun.* 254 (1999) 281–283.
- [186] K. Matsuzaki, M. Harada, T. Handa, S. Funakoshi, N. Fujii, H. Yajima, K. Miyajima, *Biochim. Biophys. Acta* 981 (1989) 130–134.
- [187] S. Ludtke, K. He, H.W. Huang, *Biochemistry* 34 (1995) 16764–16769.
- [188] K. Matsuzaki, O. Murase, H. Tokuda, S. Funakoshi, N. Fujii, K. Miyajima, *Biochemistry* 33 (1994) 3342–3349.
- [189] K. Matsuzaki, O. Murase, K. Miyajima, *Biochemistry* 34 (1995) 12553–12559.
- [190] T. Wieprecht, M. Dathe, M. Schumann, E. Krause, M. Beyermann, M. Bienert, *Biochemistry* 35 (1996) 10844–10853.
- [191] K. Matsuzaki, O. Murase, N. Fujii, K. Miyajima, *Biochemistry* 35 (1996) 11361–11368.
- [192] K. Matsuzaki, O. Murase, N. Fujii, K. Miyajima, *Biochemistry* 34 (1995) 6521–6526.
- [193] K. Matsuzaki, A. Nakamura, O. Murase, K. Sugishita, N. Fujii, K. Miyajima, *Biochemistry* 36 (1997) 2104–2111.
- [194] T. Wieprecht, M. Dathe, M. Beyermann, E. Krause, W.L. Maloy, D.L. MacDonald, M. Bienert, *Biochemistry* 36 (1997) 6124–6132.
- [195] T. Wieprecht, M. Dathe, E. Krause, M. Beyermann, W.L. Maloy, D.L. MacDonald, M. Bienert, *FEBS Lett.* 417 (1997) 135–140.
- [196] M. Schumann, M. Dathe, T. Wieprecht, M. Beyermann, M. Bienert, *Biochemistry* 36 (1997) 4345–4351.
- [197] T. Wieprecht, M. Dathe, R.M. Eppard, M. Beyermann, E. Krause, W.L. Maloy, D.L. MacDonald, M. Bienert, *Biochemistry* 36 (1997) 12869–12880.
- [198] K. Matsuzaki, K. Sugishita, N. Ishibe, M. Ueha, S. Naka-

- ta, K. Miyajima, R.M. Epand, *Biochemistry* 37 (1998) 11856–11863.
- [199] E. Jo, J. Blazyk, J.M. Boggs, *Biochemistry* 37 (1998) 13791–13799.
- [200] S.J. Ludtke, K. He, Y. Wu, H.W. Huang, *Biochim. Biophys. Acta* 1190 (1994) 181–184.
- [201] S.J. Ludtke, K. He, W.T. Heller, T.A. Harroun, L. Yang, H.W. Huang, *Biochemistry* 35 (1996) 13723–13728.
- [202] K. Matzusaki, K. Sugishita, N. Fujii, K. Miyajima, *Biochemistry* 34 (1995) 3423–3429.
- [203] E.M. Tytler, G.M. Anantharamaiah, D.E. Walker, V.K. Mishra, M.N. Palgunachari, J.P. Segrest, *Biochemistry* 34 (1995) 4393–4401.
- [204] K. Matsuzaki, Y. Mitani, K. Akada, O. Murase, S. Yoneyama, M. Zasloff, K. Miyajima, *Biochemistry* 37 (1998) 15144–15153.
- [205] A. Vaz Gomes, A. deWaal, J.A. Berden, Westerhoff, *Biochemistry* 32 (1993) 5365–5372.
- [206] Y. Pouny, D. Rapoport, A. Mor, P. Nicolas, Y. Shai, *Biochemistry* 31 (1992) 12416–12423.
- [207] E. Gazit, A. Boman, H.G. Boman, Y. Shai, *Biochemistry* 34 (1995) 11479–11488.
- [208] L. Silvestro, K. Gupta, J.N. Weiser, P.H. Axelsen, *Biochemistry* 36 (1997) 11452–11460.
- [209] J.M. Mancheno, M. Onaderra, A.M. del Pozo, P. Diaz-Achirica, D. Andreu, L. Rivas, J.G. Gavilanes, *Biochemistry* 35 (1996) 9892–9899.
- [210] G. Fujii, M.E. Selsted, D. Eisenberg, *Protein Sci.* 2 (1993) 1301–1312.
- [211] W.C. Wimley, M.E. Selsted, S.H. White, *Protein Sci.* 3 (1994) 1362–1373.
- [212] K. Hristova, M.E. Selsted, S.H. White, *Biochemistry* 35 (1996) 11888–11894.
- [213] K. Hristova, M.E. Selsted, S.H. White, *J. Biol. Chem.* 272 (1997) 24224–24233.
- [214] K. Lohner, A. Latal, R.I. Lehrer, T. Ganz, *Biochemistry* 36 (1997) 1525–1531.
- [215] R. Maget-Dana, M. Ptak, *Biophys. J.* 73 (1997) 2527–2533.
- [216] C.A. Pasternak, C.L. Bashford, K.J. Micklem, *J. Biosci.* 8 (1985) 273–291.
- [217] E.A. Groisman, C. Parra-Lopez, M. Salcedo, C.J. Lipps, F. Heffron, *Proc. Natl. Acad. Sci. USA* 89 (1992) 11939–11943.
- [218] C. Parra-Lopez, M.T. Baer, E.A. Groisman, *EMBO J.* 12 (1993) 4053–4062.
- [219] A. Perschel, M. Otto, R.W. Jack, H. Kalbacher, G. Jung, F. Gotz, *J. Biol. Chem.* 274 (1999) 8405–8410.